FUNCTION OF MULTIPLE SIGNALS IN SEXUAL SELECTION AND ANTIPREDATOR STRATEGIES IN THE SPANISH TERRAPIN, *MAUREMYS LEPROSA*

(Función de las señales múltiples en selección sexual y estrategias antidepredatorias en el galápago leproso, *Mauremys leprosa*)

Alejandro Ibáñez Ricomá

Tesis doctoral

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Memoria presentada por el licenciado Alejandro Ibáñez Ricomá para optar al grado de Doctor en Ciencias Biológicas, dirigida por el Doctor José Martín Rueda y la Doctora Pilar López Martínez del Departamento de Ecología Evolutiva del Museo Nacional de Ciencias Naturales-Consejo Superior de Investigaciones Científicas.

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El doctorando

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A mis padres

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General section

Function of multiple signals in sexual selection and antipredator strategies in the Spanish terrapin, *Mauremys leprosa*

Introduction

Communication and multiple signals

Communication is the process by which a sender transmits or interchanges information to a receiver (Davies et al. 2012). Within this theorical framework, some models have proposed that multiple different signals transmitted by male senders can be evaluated together to assess overall mate quality by the receiver or alternatively, different receivers may pay attention to different signals and to different aspects of male quality (Reviewed in Candolin 2003). For example, in male ostriches different traits may inform about different components of the immune system, suggesting that multiple signals in sexual selection reflects different aspects of individual quality (Bonato et al. 2009). Alternatively, signal reception by the receivers might be favoured by producing different components in different sensory modalities, enhancing their detectability, discriminability and memorability by the receivers (Rowe 1999).

Animals live in different habitats influenced by several physical constraints that may shape their sensory systems to optimize their communication abilities (Wehner 1997). In this way, animal communication may involve a wide range of sensory channels. The main sensory modalities include tactile, chemical, visual and auditory systems, but some fish may produce and detect electrical fields (Halliday & Slater 1983).

Chemical signals

Chemical signals are commonly used by many animals during social and sexual behaviour (Wyatt 2003; Mason & Parker 2010). Reptiles are not an exception, so intraspecific chemical communication has a crucial role in mate searching and mate selection, as well as in discrimination of the quality of potential partners (Cooper & Vitt 1984, 1986; Mason 1992; Martín & López 2000, 2011; Mason & Parker 2010). For example, femoral glands in males of some species of lizards emit odours that may contain specific information about the traits of the signaller and, thus, females may base their choice on such odours (Olsson et al. 2003; López & Martín 2005; Martín & López 2006, 2011). Chemical signals are also important in intrasexual relationships between males, where they can contribute to reduce costs of aggressive interactions (Carazo et al. 2007; Martín & López 2007; López & Martín 2011). For example, male Iberian rock lizards (*Podarcis hispanica*) are less aggressive toward males impregnated with scent of females, suggesting that female-like scent indeed reduces the aggressive response of territorial males (López & Martín 2002). Similarly, male Iberian rock lizards avoid scent marks from unfamiliar conspecific because the probability of success in an agonistic encounter with an unknown male is low (Aragón et al. 2001a, b, 2003).

Many studies have demonstrated that turtles have very well-developed nasal and vomeronasal systems (Halpern 1992; Hatanaka & Matsuzaki 1993; Fadool et al. 2001) that are mainly used to make discriminations of many different scents, suggesting a good ability of turtles to detect chemical cues in the environment. For example, female Painted turtles (*Chrysemys picta*) use chemical cues solved in the water of the ponds where they lived for homing behaviour and to occupy the water of their original ponds (Quinn & Graves 1998). Mental glands are integumentary organs consisting of simple

or branched invaginations of the epidermis on the skin of the throat, that are largely common distributed in freshwater turtles, above all, in the family Geomydidae (Table 1, next page). These glands are active during the breeding season only (Rose et al 1969) and the chemical composition of their secretions may suggest that mental gland secretions serve as an olfactory cue during courtship and/or male combat (Rose 1970). In agreement, recent laboratory experiments have showed the importance of inter-and intrasexual chemical communication in turtles (Muñoz 2004; Poschadel et al. 2006; Galeotti et al. 2007; Lewis et al. 2007; Polo-Cavia et al. 2009). Males of some freshwater turtles can discriminate pheromones of other males solved in water, and use this information to avoid encounters and agonistic interactions with potential competitors (Poschadel et al. 2006; Polo-Cavia et al. 2009). Similarly, male and female *M. leprosa* terrapins tend to avoid water with chemicals cues from conspecifics of the opposite sex outside of the mating season, probably to avoid aggressive interactions between sexes (Muñoz 2004). In addition, during the mating season, males prefer occupying water with chemical cues of females and females prefer water that contains chemicals from other females (Muñoz 2004).

Visual signals

Among lower vertebrates, coloration depends on the presence of pigmented cells called chromatophores (Cooper & Greenberg 1992; Grether et al. 2004). Dermal chromatophores are composed of different layers: the xantophore is yellow to red colour and contains pteridines or carotenoids absorbing at short wavelengths; the iridiphore iridescent or silvery containing layers of guanine rodlets that produce structural colours; the melanophore is brown to black and contain melanin which absorb

Family	Genera	Presence of mental glands	Habitat
GEOEMYDIDAE			
	Batagur	+	Aquatic
	Callagur	-	Aquatic
	Chineyms	+	Semiaquatic
	Cuora	+	Aquatic/Semiaquatic
	Cyclemys	+	Semiaquatic
	Geoclemys	+	Aquatic
	Geoemyda	+	Terrestrial
	Hardella	-	Semiaquatic
	Heosemys	+	Semiaquatic/Terrestrial
	Hieremys	+	Semiaquatic
	Kachuga	-	Aquatic/Semiaquatic
	Malayemys	-	Aquatic
	Mauremys	+	Aquatic
	Melanochelys	+	Aquatic
	Morenia	+	Aquatic
	Notochelys	+	Semiaquatic
	Ocadia	+	Aquatic
	Orlitia	+	Aquatic
	Rhinoclemys	+	Aquatic/Semiaquatic/Terrestrial
	Sacalia	+	Semiaquatic
	Siebenrockiella	+	Aquatic
EMYDIDAE			
	Chrysemys	-	Aquatic
	Clemmys	+	Aquatic/Semiaquatic/Terrestrial
	Deirochelys	+	Aquatic
	Emydoidea	-	Aquatic
	Emys	-	Aquatic
	Graptemys	-	Aquatic
	Malaclemys	-	Aquatic
	Pseudemys	-	Aquatic
	Terrapene	-	Terrestrial
PLATYSTERNIDAE			
	Platysternon	+	Semiaquatic
TESTUDINIDAE			
	Chersina	-	Terrestrial
	Geochelone	+	Terrestrial
	Gopherus	+	Terrestrial
	Homopus	-	Terrestrial
	Kinixys	-	Terrestrial
	Malacochersus	-	Terrestrial
	Psammobates	-	Terrestrial
	Pyxis	-	Terrestrial
	Testudo	-	Terrestrial

Table 1. Presence (+) or absence (-) of mental glands in 40 genera of the families Geomydidade, Emydidae, Platysternidae and Testudinidae, based in Berry & Shine 1980; Winokur & Legler 1975.

light throughout the visible spectrum and ultraviolet range wavelength, and thus producing a dark integumentary coloration (Cooper & Greenberg 1992; Grether et al. 2004).

In many species of vertebrates body coloration is involved in sex recognition or rival assessment (Baker & Parker 1979; Cooper & Greenberg 1992; Andersson 1994). Ornamental colours have been deeply studied in birds, where carotenoid and melanin pigments may convey information of different traits (McGraw & Hill 2000). In one hand, carotenoid pigments are often related with nutritional status and health state parameters, such as immune system and parasite resistance (Hill 1990; Hill et al. 1994; McGraw & Hill 2000; Mougeot et al. 2009). On the other hand, melanin-based colours mostly reflect fighting ability or dominance in males. Reptiles display conspicuous colorations during the breeding season that might be important for partner selection in many species of lizards (reviewed in Cooper & Greenberg 1992). For example, inter-individual variation in male *Lacerta schreiberi* lizards' coloration can be related to some traits, such as body size, body condition and immune response that may be important for mate choice (Martín & López 2009). Similarly, in striped plateau lizards (*Sceloporus virgatus*), different components of colour ornamentation such as area and chroma may reflect different aspects of the phenotypic quality of the female and her offspring, suggesting a condition-dependent signal to potential mates (Weiss 2006; Weiss et al. 2011). In addition, coloration can play an important function in agonistic interactions between males. For example, male tree lizards (*Urosaurus ornatus*) with larger blue spots dominated over males having smaller blue spots in competition for a limiting resource (Thompson & Moore 1991). In the same way, throat UV coloration

was an important predictor of male fighting abilities in male flat lizards, *Platysaurus broadleyi* (Whiting et al. 2006).

Several studies have demonstrated the existence of sexual dichromatic coloration in some turtles. For example, males of *Batagur baska* and *Callagur borneoensis* change the colour of their head and shell during the breeding season. Male spotted turtles (*Clemmys guttata*) have darker skin at the chin region than females suggesting that coloration might be a sexually selected trait (Rowe et al. 2013). Adult males of the northern map turtle (*Graptemys geographica*) express brighter, richer and more yellow-shifted coloration of their postorbital head spots than adult females (Bulté et al. 2013). However, only a few recent studies have shown that colourful skin patches and stripes of the head of turtles may be linked with individual quality, suggesting that coloration could evolve under sexual selection pressures in *Testudo hermanni* tortoises (Galleotti et al. 2011) and in *Trachemys scripta* terrapins (Polo-Cavia et al. 2013). Also, male tortoises with darker shells were more aggressive toward conspecifics, suggesting that melanin-based coloration may be a signal of aggressiveness (Mafli et al. 2011). Similarly, many other turtle species have colourful stripes in limbs and head that could be involved in sexual selection (Ernst & Barbour 1989; Figure 1). Nevertheless, the stripes of the head and limbs of freshwater turtles could have an additional cryptic function rather than sexual signalling, and help to break up their outlines against vegetation when viewed by a predator from above or below.

Figure 1. Conspicuous colorations of different turtle species: Testudo hermanni (top left photo), Emys orbicularis (top right photo), Mauremys leprosa (bottom left photo), Podocnemis unifilis (bottom right photo). Picture credit: Alejandro Ibáñez and José Martín.

Predation risk as a cost of sexual signals and reproduction

Sexual signals are directed to find a mate with the benefit of reproduction, enhancing the fitness of the sender. However, during sexual signalling, the individuals are also more vulnerable to be localized by enemies due to their greater detectability intended to receivers (reviews in Magnhagen 1991; Zuk & Kolluru 1998). In this framework, several studies have shown that signals addressed to conspecifics for inter- and intrasexual communication may be exploited by predators in many animals. For example, calls of male petrels (*Halobaena caerulea)* emitted in order to attract mates can be used by a local predator as a cue for location and selection of them as preys

(Mougeot & Bretagnolle 2000). Similarly, brighter lizard models were more conspicuous to avian predators and were detected and attacked significantly more times than duller ones (Stuart-Fox et al. 2003). Also, sharptooth catfish (*Clarias gariepinus)* have well-develop electroreceptors that use to detect the pulse signals of their main preys (Hanika & Kramer 2000).

Concerning reproduction, the trade-off between current and future reproductive investment may produce a high cost for individuals (Reznick 1985; Roff 1992; Stearns 1992). Costs of reproduction may incur in fecundity, survival or both (Bell 1980;Shine 1980; Brodie 1989; Landwer 1994). In addition, there is an increased susceptibility to predation associated with all stages of reproduction (reviewed in Magnhagen 1991). In some cases reproductive females are more exposed to predators during the breeding season due to behavioural and physiological changes related with reproduction. For example, female Mallards experience their highest tax of mortality in the breeding season, especially during the incubation of the nests when they are exposed to terrestrial predators (Arnold et al. 2012). Similarly, gravid female lizards bask more time due to their higher thermoregulatory demands assuming a riskier behaviour than non-gravid ones (Shine 1980). In some species of lizards, gravid females showed a locomotor decreased capacities that might compromise their escape strategies when facing a predator. For example, gravid females show a detriment in their sprint speed or endurance (Bauwens & Thoen 1981; Sinervo et al. 1991; Van Damme et al. 1989).

In spite of increased predation risk, individuals with extravagant ornamentation, as well as those in a gravid reproductivecondition, might behaviourally compensate by acting more prudently. For example, field cricket (*Gryllus integer*) males with longer calling bouts behave more cautiously when are introduced in a novel environment and after disturbance than those males with short calls (Hedrick 2000). Also, more

conspicuous individual male *Iberolacerta monticola* behave shier, hiding for longer in refuges when they are threatened by a potential predator (Cabido et al. 2009). Similarly, reproductive individuals should take fewer risks due to their higher immediate fitness (Clark 1994). Also, behavioural compensation may occur in response to constrains in locomotor performance during reproduction. For example, gravid females can rely more on crypsis (Brodie 1989; Bauwens and Thoen 1981; Cooper et al. 1990) or switch their antipredator strategy (Husak 2006). Thus, reproductive individuals as well as those individuals with exaggerated sexual traits might not always experience a greater actual predation through switching their antipredator strategies.

Antipredator behaviour

Natural selection by predation is one of most important selective forces that impose high costs for the behaviour of individuals in a number of vital activities such as foraging and reproduction (reviewed in Lima & Dill 1990). Given the direct benefits for the prey by reducing mortality, an optimal antipredator strategy facing a predator should also take into account the costs in terms of time and energy allocation with important fitness consequences, derived of an exaggerated antipredatory response (Lima 1998; Rhoades & Blumstein 2007). However, animals use compensatory behavioural mechanisms to reduce the costs linked to antipredator strategies. For example, frequent use of refuges may lead to a deteriorated body condition in lizards (Martín & López 1999a), but individuals can compensate for this cost with modifications in their hiding behaviour (Amo et al. 2007).

In some ecosystems situated in temperate regions, the main hazard for freshwater turtles comes from terrestrial predators, basically mammals and birds

(Martín & López 1990). Turtles usually bask on emerged rocks or deadwood surveys remaining on the surface of the water, and when they detect any approaching potential predator, they quickly dive back in the water (López et al. 2005a). However, this antipredator strategy is worthless when turtles are away from water and more vulnerable to be intercepted by terrestrial predators. For example when looking for nesting places, travelling between different water bodies, or during drought periods when temporary water courses dry out (Andreu & López-Jurado 1998). Thus, turtles should use other strategy to deter predators in the terrestrial habitats. In this context, turtles have morphological structures such as the shell that offers partial protection (i.e. refuge) until turtles can escape to a safer refuge deep in the water. Therefore, turtles can withdrawn and hide the legs, head and tail inside the shell when a predator approaches too close and/or try to attack them (Greene 1988; Hugie 2003; Martín et al. 2005).

However, increasing the time spent hiding in a refuge, or into the shell, may entail a loss of feeding and/or mating opportunities (Ydenberg & Dill 1986; Sih 1997; Martín et al. 2003a, b; Reaney 2007) or thermoregulatory costs (Martín & López 1999a, b; Polo-Cavia et al. 2009). Therefore, the decision of when to emerge from the refuge and initiate an active escape may lead to a trade-off that animals should be able to optimize by modifying hiding times (Sih 1997; Martín & López 1999b; Polo et al. 2005; Cooper & Frederick 2007). Hiding times tend to increase with the costs of emerging attributable to predation risk, suggesting that the ability to assess the risk level is essential to decide when to leave the refuge (Scarratt & Godin 1992; Martín & López 1999b, 2001a, 2005; Cooper 2009).

The balance between the costs of remaining hidden vs. costs of emergence should also depend on several traits such as body size (e.g. Krause et al. 1998; Martín & López 2003), conspicuousness of sexual coloration (e.g. Cabido et al. 2009; Journey et

al. 2013), reproductive state (e.g. Frommen 2009) or health state (López et al. 2005b). These factors may explain the inter-individual variations in boldness under predation risks, which have often been used to define different personality types (e.g. Riechert & Hedrick 1993; López et al. 2005b; Jones & Godin 2010). In fact, natural selection, through the predation regime, may be one of the main forces modulating the evolution of personality traits (Bell & Sih 2007; Digenmanse et al. 2007).

Firstly, the interactions between prey and predator may be influenced by their relative body size (Lundval et al. 1999; Scharf et al. 2000; Aljetlawi et al. 2004). Prey size may involve other factor that could influence boldness under predation risk in opposite ways. In one hand, there are many examples supporting the idea that larger individuals should need longer hiding times after a predator attack. For example, locomotor and righting performance might be constrained by body size in turtles (Bonnet et al. 2001, 2010) and consequently their ability to escape actively, which in turn should increase the amount of time spent hiding in the shell after being attacked by a predator. In addition, larger turtles could be slower (e.g. lesser rate of limb movement) and more conspicuous to predator eyes than small ones. Similarly, in insects, an increase in body size may make the individual more likely to be found and/or attacked by birds (Mänd et al. 2007).

On the other hand, small individuals might be more defenceless and consequently act shier in front of a predator attack. For example, vulnerability in juveniles of some fish species depend of the relation prey/predator length suggesting that the smallest sizes are more susceptible (Scharf et al. 1998). Similarly, smaller hatchling turtles have a higher predation rate than larger ones (Tucker et al. 1999; Janzen et al. 2000). This could force small turtles to remain hiding inside their shell for

a longer time, in order to ensure that any predators in the surrounding area will have most likely gone away.

Similarly, predation risk might be affected by the degree of showiness expressed by individuals because it affects the conspicuousness of prey to predators. For example, brighter and more UV-reflecting sexual ornaments may increase the probability of the individual being detected by raptors (Viitala et al. 1995; Honkavaara et al. 2002). Thus, in the absence of benefits related with showy coloration, natural selection should favour inconspicuousness against visual backgrounds (Endler 1984; Merilaita et al. 1999; Martín & López, 2001b). Nevertheless, sexual selection through intra-sexual competition and inter-sexual selection enhances the degree of conspicuousness in secondary sexual traits such as colour ornaments favouring showy individuals (Andersson 1994). For example, female mate choice may favour colourful ornamentation in males as an honest signal (Kodric-Brown & Brown 1985; Houde 1987; Hill 1991; McGraw & Ardia 2003). Similarly, female coloration might reflect different aspects of phenotypic quality and could be implicated in male mate choice (Amundsen 2000a, b; Amundsen & Forsgren 2001; Weiss 2006; Weiss et al. 2011). Recently, some studies with chelonians showed that colourful patches and stripes on the head and limbs might act as honest visual signals that could imply some traits that are important in intra-sexual competition and/or mate choice (Galeotti et al. 2011; Polo-Cavia et al. 2013). Colourful stripes on the head and limbs in turtles can be completely or partially hidden from predators' view by remaining withdrawn into the shell, whose usual cryptic coloration suggests that coloration might be modulated by both natural and sexual selection forces. Antipredatory behavioural responses should be constrained by individual showiness. Thus, individuals with more striking coloration should be shyer in

risky situations in order to avoid being easily detected (Cabido et al. 2009; Journey et al. 2013).

Another factor that might affect boldness under risk of predation is the reproductive state of an individual. Optimality models predict that prey with greater initial fitness should be more cautious against predators (Cooper & Frederick, 2007). This matches the "asset-protection principle" (Clark 1994) that predicts that individuals with lower fitness tend to take higher risks than those with higher fitness that take fewer risks, which would become asset protecting. Thus, gravid females might act shier due to their higher immediate fitness value than non-gravid ones. In addition, some studies have suggested a locomotor impairment of gravid females due to the additional weight of the clutch (Shine 1980; Van Damme et al. 1989) or physiological changes (Brodie 1989; Olsson et al. 2000). Gravid females may also shift their behaviour independently of an increased weight (Cooper et al. 1990; Schwarzkopf & Shine 1992). Along the same lines, because, in reptiles, body temperatures before ovoposition influence developmental rates as well as phenotypic traits of the resultant offspring (Shine 2006), gravid females require basking at higher frequencies and for longer periods for reaching optimal temperatures for a correct embryo development (Braña 1993; Blázquez 1995; Brent Charland & Gregory 1995). On the other hand, energy reserves are allocated during reproductive season of females and might be important in terms of fecundity (Doughty & Shine 1998). Metabolic rates tend to increase during gestation in pregnant female lizards due to embryogenesis (Angilletta & Sears 2000; Robert & Thompson 2000). In the same way, egg load occupies a great part of body cavity that might compress internal organs as lungs incurring in high energetic cost of breathing (Munns 2013). Thus, switching boldness in risky situations associated to gravidity in females

might be also driven by thermoregulatory, metabolical and physiological requirements derived of egg production.

Objectives

The main goal of this thesis was to study the evolution and functionality of multiple signals (chemical and visual) in sexual selection processes (mate choice and male competition) and their influence on the optimization of antipredator strategies in the Spanish terrapin (*Mauremys leprosa*). To accomplish this, the thesis is organized in six chapters with the following concrete objectives:

Chapter I. This study examined the behavioural responses of Spanish terrapins to chemical cues of conspecific individuals of different body size and health state. The first experiment aimed to examine the potential role of chemical cues in partner choice. For this, a laboratory experiment was performed where each turtle could choose between a pool with clean water and a pool with water with chemical stimuli from individuals of the opposite sex. Thus, the first part of this study tested whether turtles were able to discriminate and showed preferences for some individual traits that may be important in mate choice, such as body size or health state, through chemical cues alone emitted by conspecifics of the opposite sex. In a second experiment, the behavioural responses of males to chemical cues of other males were examined in an experiment where each male could choose between a pool with clean water and a pool with water containing chemical stimuli from other males. We predicted that size and relative size of male pairs should be the main and most important factor in agonistic interactions between males. In this case, males should avoid occupying water with chemical cues of larger males to reduce the risk of intrasexual aggression, and should prefer occupying water with chemical stimuli of smaller males, which may successfully attack to increase the probability of mating with females living in the area.

Chapter II. This study examined whether male *M. leprosa* turtles were able to recognise and distinguish familiar from unfamiliar males via chemical cues released to water, and whether male turtles may modify their space use based on this information alone. We designed a laboratory experiment similar to that of the previous chapter. We predicted that males should avoid pools containing water with chemicals from unfamiliar males respect to pools with water with chemicals from familiar males or with water with their own odour. This study also analysed the effects of body size and boldness of male turtles on their response to chemical cues of familiar and unfamiliar conspecific males. Large unfamiliar males, but not small unfamiliar males, could represent a potential dangerous situation during competition for resources or agonistic interactions between males and, thus, large males should avoid the chemical cues from large unfamiliar males but not the chemical cues from large familiar males or their own odour. In contrast, small males might not need to avoid the odours from other small unfamiliar or familiar males, because aggression levels between small males would be low. Concerning boldness, shy males should become subordinate, or at least, less successful in competition than bold males. Thus, bold male turtles should avoid the chemicals from unfamiliar males but not from familiar males because they could have established a previous hierarchy with them and these bold turtles would be very likely dominant. In contrast, shy turtles should avoid the chemicals from any kind of male because any male is likely to be dominant on shy males.

Chapter III. This study explored the existence of sexual dichromatism in different colourful traits (shell and limb stripes) of Spanish terrapins. Both males and females have orange-yellow stripes at head, neck and limbs, which could act as honest signals in intrasexual competition and mate choice. We expected that these colourful stripes should show intersexual differences if coloration is under different sexual

selection pressures in males and females. However, shell colour, which is typically brown-green, should not show variation between sexes because it is exposed directly to predators, and should evolve to be equally cryptic in both sexes. This study also examined the relationship between characteristics of coloration of the shell and limb stripes with body size and health-related physiological parameters (i.e., body condition, haematocrit and immune response) that could be related with dominance or quality of potential mates. We expected that female and male turtles could also visually signal for different messages to conspecifics though the characteristics of colourful ornaments which could be related to body size and health state of the signaller.

Chapter IV. This study explored how sex, body size and sexual coloration of Spanish terrapins affected inter-individual variability in boldness, as reflected by the antipredator hiding behaviour of withdrawing head and limbs into their own shell. Turtles were subjected to simulated predatory attacks with different levels of risk. Typically, there are two components of the hiding response of the turtles: the time that the turtles spent withdrawn entirely into the shell until the head is out of the shell (i.e. appearance times), and the subsequent time after emergence from the shell that the turtles spent immobile monitoring for predators before starting to escape actively (i.e. waiting times). We predicted that boldness should depend on individual traits as well as on risk level. However, inter-individual variation in gender, size and coloration should have relatively less importance when risk level increases because antipredator behaviour under high risk should be more conservative in all individuals.

Chapter V. This study explored whether reproductive state of female terrapins *M. leprosa* imposes a cost in their boldness (i.e. antipredatory hiding responses into their shells) and/or exploratory behaviour. Gravidity in reptiles may have costs in terms of performance, thermoregulation and/or metabolism. In addition, gravid females have

higher current reproductive value and, thus, they should act more cautiously than non-gravid ones. As in the Chapter IV, turtles were subjected to simulated predatory attacks with different levels of risk. In addition, the exploratory activity of female turtles was monitored in a novel-environment test. We predicted that the costs derived of egg production and embryo maintance together with the higher current reproductive value of gravid females should render them shier under predation risk and less exploratory in novel environments.

Chapter VI. This study explored in the field the relation of basking patterns of male terrapins, *M. leprosa*, with health state, body size and limb colour showiness. Because basking behaviour might have important benefits in terms of parasite skin protection, we expected that those individuals with higher basking activity should have better health state than those with lower basking activity. In addition, larger turtles should need longer basking times to afford their slower heating rates. However, basking behaviour might be risky because the main predators in the study site come from terrestrial habitats. Thus, we predicted that turtles with greater skin colour conspicuity might be more easily detectable by potential predators while basking, and should, therefore, reduce basking activity to decrease predation risk.

Material and methods

The general methods of this thesis are described in this section, which chiefly focuses on the species and site of study as well as the procedure used to capture, husbandry and taking measurements of the turtles. A more detailed description of the methods used in each experiment will be found in each chapter.

Study species: The Spanish terrapins

The Spanish terrapin *(Mauremys leprosa* Schweiger 1812) is a thermophile medium size freshwater species, which is distributed in southwestern Europe and northwestern Africa (Busack & Ernst 1980; Andreu & López-Jurado 1998; Keller & Busack 2001; Da Silva 2002) (Figure 2). In the Iberian Peninsula is much more common in the southern half, scarce in the northern plateau, and very rare in the north, with isolated populations in scattered points (Gosa & Bergerandi 1994). This turtle preferably inhabits ponds and streams with riparian vegetation, being less common in large rivers and reservoirs. This species is very resistant to brackish water, and pollution, but not excessive (Andreu and López-Jurado 1998, Da Silva 2002).

The shell is olive-brown and the plastron yellow or cream with dark spots (Figure 3). This species has orange stripes on the head, neck and legs (Andreu & López-Jurado 1998) that are more intense in juveniles, followed by adult females and adult males, which are less colourful

Figure 2. Original distribution area of M. leprosa *based in Iverson (1992).*

(personal observation). Females reach larger sizes than males and have a plastron which is less concave than that of males (Figure 3). Males sexually mature at seven years old, with about 13 or 14 cm of carapace length, while females acquire maturity at an age of 10 years, about 14 or 16 cm long (Pérez et al. 1979). The breeding season occurs in spring, during the months of May and June, and females can lay between 1 and 13 eggs (Andreu & López-Jurado 1998; Arnold & Ovenden 2002). These turtles can live 20 years in captivity. The diet consists of insects, small molluscs and crustaceans, amphibians, fish, plants and carrion (Salvador 1985; Crespo & Oliveira 1989). This turtle is listed as a "vulnerable" species according to IUCN criteria (Da Silva 2002).

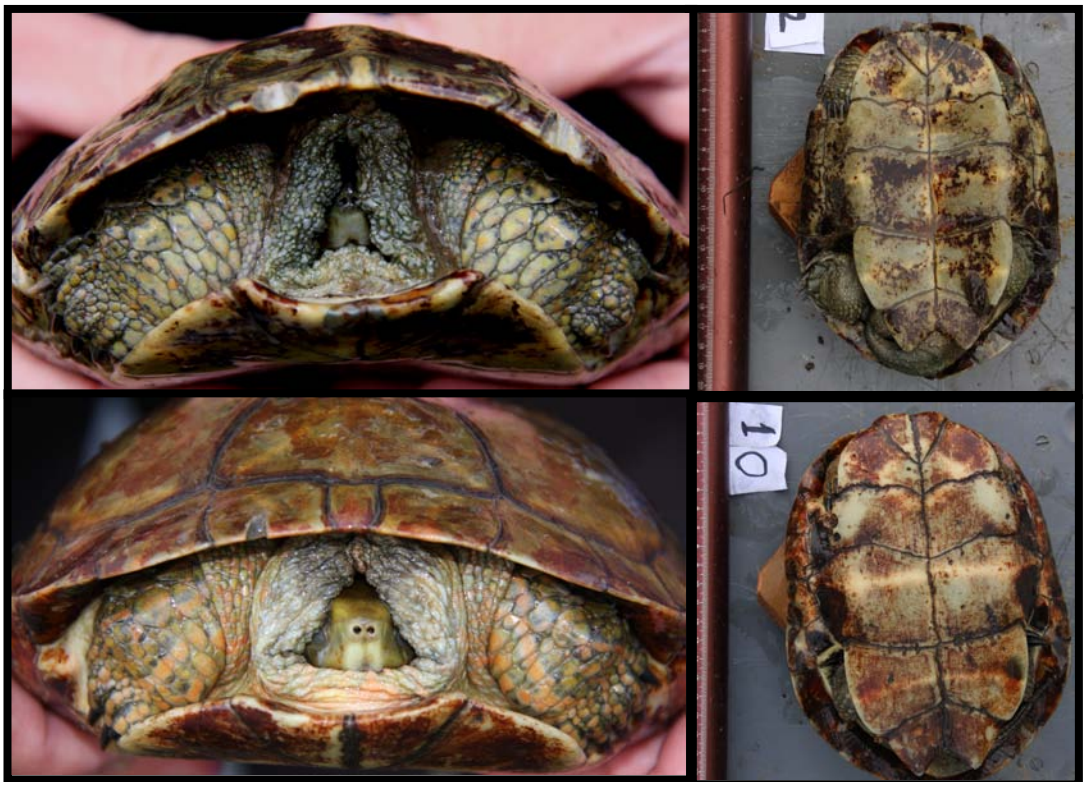

Figure 3. Sexual dimorphism in M. leprosa*. Adult male on the top, and adult female on the bottom. Picture credit: Alejandro Ibáñez and José Martín.*

Study area

The study was carried out in a dehesa woodland at Olivenza and Alconchel (Badajoz Province, Southwestern Spain) at an elevation range of 200-400 m. This agroforesty system is characterised by the presence of a savannah-like open tree layer, mainly dominated by Mediterranean evergreen oaks – holm oak (*Quercus ilex*) and cork oak (*Quercus suber*) - with an herbaceous layer comprised of either cultivated cereals (oats, barley, wheat) or, more commonly, native vegetation dominated by annual species, which are used as grazing resources (Joffre et al. 1999). The weather in this region responds to a subtropical Mediterranean climate, showing an approximate average annual temperature and rainfall records of 16 ºC and 500 mm, respectively. However, these dehesa woodlands in southwestern Spain are subjected to high rainfall unpredictability with long periods of drought during summer (Joffre et al. 1999).

The study area is located in the basin of the Guadiana River with several tributary small streams, and there are also numerous isolated small to medium sized semi-artificial ponds used for watering livestock. All freshwater habitats in the area hold a substantial population of turtles.

Turtle capture

A modified version of the traditional underwater funnel traps was used to capture the turtles in their freshwater habitats. These traps have two funnel-shaped entrances on the bottom and include a mesh chimney that reaches from the body of the trap to the surface allowing the turtles to come to the surface to breath (Kuchling 2003; T & L Netmaking, Mooroolbark, Victoria, Australia) (Figure 4). Turtles were attracted by the smell of the

bait (i.e. sardines) that was held into a perforated container suspended inside. To prevent the turtle from drowning, every trap was revised every hour to collect all turtles inside. All collected turtles were alive and did not show any sign of being stressed inside the traps.

Figure 4. One of the traps used to capture the turtles. Picture credits: José Martín.

Animal husbandry

Turtles were held in captivity in a country house building close to the capture sites while we took morphological, coloration and physiological measurements (see below) and to be used in other experiments. Then, some turtles were transported in plastic cages (80x40 cm and 50 cm height) to "El Ventorrillo" Field Station, near Navacerrada (Madrid province) where experiments were conducted. The journey was made by car with a duration of four hours while maintaining a constant temperature of 23 ºC to avoid heat stress. We did not observe any effect of transport on mortality or health state of the turtles. Turtles were housed in individual outdoor plastic aquaria (60 x 40 cm and 30 cm height) containing water and rocks that allow turtles to bask out of water. The photoperiod and temperature were the same as those of the surrounding area. Turtles were fed three times a week minced meat, earthworms, and a commercial compound of turtle "pellets". Turtles were held in captivity and the investigator minimised contact with the animals before and during all behavioural experiments to avoid possible effects of habituation. All individuals were healthy and in good condition during the tests.

When the experiments were finished, turtles were returned to the exact locations of capture.

Body size estimation and health-physiological parameters

We used a metal ruler to measure to the nearest millimetre the body size (shell) of turtles: maximum carapace length was measured as the greatest straight-line distance from the anterior end to the posterior end of the shell. We used an electronic balance to measure body weight. In addition, we calculated the residuals of the regression between weight and body length (both log-transformed) as a measure of body condition of individuals (Polo-Cavia et al. 2010).

To assess the immune response of turtles we used the phytohaemagglutinin test (PHA) (Smits et al. 1999; Belliure et al. 2004; Tella et al. 2008). This is a test “in vivo” that estimates the delayed hypersensitivity immune response mediated by T lymphocytes (CMI), but that may also involve components of innate and adaptive immune system (Martin et al. 2006; Tella et al. 2008). Nevertheless, we used this index because it is a standard measure of immunocompetence and health state, regardless of the type of cells involved in it (Polo-Cavia et al. 2010). We used a spessimeter (accuracy of 0.01 mm) to measure the thickness at the same marked point of the left hind paw of turtles before and 24 h after injection of 0.04 mg of PHA dissolved in 0.02 ml of phosphate buffered saline (PBS). General health of turtles was not affected by this test. The only noticeable effect of the injection of PHA was a slight swelling of the skin that disappeared after 48 h. We calculated the immune response (CMI) as the difference between the thickness of the leg at the injection site before and after antigen injection (Belliure et al. 2004; Martin et al. 2006; Polo-Cavia et al. 2010).

Blood samples were collected from the caudal sinus at the base of the tail by using a 1 mL syringe, with a 30 G, 1/2, 0.3 x 13 mm heparinised needle for each turtle (Polo-Cavia et al. 2010). Blood was placed in a heparinised capillary tube and centrifuged during 10 min at 10,000 rpm. The haematocrit (i.e., the volume percentage of red blood cells in blood) was measured with a graphical scale in each capillary tube as the ratio between the length of the capillary tube occupied by packed red blood cells and the total length of the capillary tube occupied by the blood sample. We also prepared a smear on a microscope slide from the blood taken. Blood smears were air-dried, fixed in absolute ethanol for 10 min, and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) for 40 min before their examination. Blood smears were used to estimate the total numbers and the proportions of different types of leucocytes under magnification, ×1000. The proportions of different types of leucocytes were assessed on the basis of an examination of a total of 100 leucocytes. The number was quantified as number of leucocytes per 100 fields examined. The heterophile:lymphocyte (H:L) ratio was estimated from the percentages of heterophiles and lymphocytes per 100 leucocytes obtained in these counts (Merino et al. 1999).

Colour measurements

Reflectance spectra between 300 and 700 nm were recorded using an Ocean Optics UV-VIS (JAZ-EL200) spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA) on two traits (limb stripes and shell) of each turtle. We recorded this range of reflectance, including UV range (300-400 nm), because freshwater turtles have UV receptors that allow a tetra-chromatic colour vision (Arnold & Neumeyer 1987). Mean reflectance was summarized over 5 nm steps (“binned”, Grill & Rush 2000). To the naked eye, limb and

neck stripes appear uniformly yellow-orange in limb and brown-green in the shell. Turtles have retractable necks and usually hid the head inside the shell when handled. Thus, due to the difficulty of measuring coloration in the neck stripes of turtles and to prevent harming turtles, we measured coloration at five haphazardly selected places of the forelimbs orange stripes (right and left) and at five haphazardly selected places of the dorsal part of the shell (i.e., carapace) trying always to measure the same or similar body points in all individuals. Then, we calculated a mean for the limbs and other for the shell, and used these values for further analyses. Reflection was recorded using a optic fiber probe with a core diameter of 400 μm (QR400-7-UV/ VIS-BX; Ocean Optics Inc.) held at a 90° angle and at 5 mm from the surface. Measures of reflectance were always made by the same person (AI). A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

Basking behaviour of turtles

We used plastic labels with alphanumeric codes (Figure 5) for long-distance detection and individual identification. To mark the turtles on the dorsal part of the shell with the plastic labels, we first used sandpaper to remove irregularities of the second vertebral shell scute and, then, we fasten the plastic label with epoxy glue to the scute. Subsequently, turtles were placed in individual plastic boxes and left there overnight. The day after, turtles were released to their origin ponds for monitoring their behaviour during the next months. We checked the behaviour of the turtles using binoculars and a telescope watching from a hidden position near the pond.

The same person (AI) monitored turtles basking activity during 19 days divided between the months of April and May. The observations were made between 10.00 AM and 19.30 PM covering a wide-range of freshwater turtle daily activity periods (Rowe & Moll 1991). We made observation in different periods of the day that ranged between 20 and 290 min. In every period of observation we tried to identify the maximum number of turtles through the plastic labels. Then, we monitored the behaviour of each turtle identified till the end of the period or till it was not visible anymore (i.e. dive into water). We considered that a turtle was basking when it was remaining immobile out of the water, exposed to solar radiation directly or solar heated surfaces, such as shorelines, rocks or deadwood logs. Thus, we noted down the time devoted to basking by each turtle. Then, we determined a specific basking activity index for each individual, expressed as the ratio between the basking time of the turtle and the overall monitoring time for the pond.

Figure 5. Plastic labels with alphanumeric codes used for long-distance detection and individual identification. Picture credits: Alejandro Ibáñez and José Martín.

Results and discussion

Role of chemical cues in mate choice and male intra-sexual recognition

Spanish terrapins were able to distinguish through chemical cues alone some traits of conspecifics that could be important in sexual selection and the presence of these chemical cues affected their selection of determined water pools. Thus, the results obtained in **Chapter I** suggested that chemical signals may be involved in mate choice. However, female and male turtles had different responses when exposed to chemical cues of different conspecifics of the opposite sex. Females showed a preference for water pools with chemical cues of heavier males but the body condition or immune response of the male donor of the chemical cues were not important for their choice (Table 3). Forced inseminations are a common mating strategy in aquatic turtles (Berry & Shine 1980). Female mate choice for larger males also occurred in the lizard *Ameiva plei*, where large males may be better equipped to protect females of the harassment of other males allowing females increasing foraging time (Censky 1997). Thus, female may choose pools with water with chemical cues of large males, because these males might offer protection against other males living in the place, thereby increasing their lifetime reproductive success. Otherwise, male *M. leprosa* preferred to occupy pools containing water with chemicals of healthy females (i.e. greater immunocompetence), but pool selection did not depend of the body size and condition of the female donor of the chemical cues (Table 3). In other animal species, males may signal their health state through pheromones allowing female choice for healthier males (Penn & Potts 1998; Rantala et al. 2002, 2003; Martín & López 2006). Female *M. leprosa* with better immune responses should have individual greater resistance to parasites or diseases

(Hamilton & Zuk 1982), which can be inherited by their offspring (Zahavi 1975; Andersson 1994). Thus, male terrapins could adopt a mate choice strategy where they can enhance their fitness through increasing offspring quality and survival.

	Time spent in water with chemical cues by:			
Traits of the donor	Females		Males	
of the chemical cues	$F_{1,37}$	P	$F_{1,35}$	P
Weight	**6.88**	**0.017**	0.40	0.54
CMI response	0.05	0.82	**6.12**	**0.03**
Body condition	0.14	0.71	0.89	0.36

Table 3. Relationship between the time spent by female or male turtles in water pools with chemical cues of a conspecific of the opposite sex and the individual characteristics of the subjects acting as the donor of those chemical stimuli. Significant effects (P < 0.05) are indicated in bold.

Concerning male interactions, **Chapter I** and **Chapter II** highlighted the importance of body size in chemosensory recognition between males. Male *M. leprosa* avoided water with chemicals of conspecific larger males, but oriented toward water with chemicals of smaller males. In addition, large but not small males spent significantly less time in the pools with scent of unfamiliar males than in pools with scent of familiar males or with their own chemicals, which did not significantly differ suggesting that only large unfamiliar males might represent a potential danger. In agreement, in the turtle *Emys orbicularis*, males avoid the odours of larger males and prefer to occupy water with chemical stimuli from relatively smaller males (Poschadel et al. 2006). Similarly, during basking activity in wild populations, larger turtles displace smaller turtles more often than the converse (Bury & Wolfheim 1973; Bury et al. 1979; Lovich 1988; Lindeman 1999). Taken together, these results suggest that

relatively smaller males attempt to avoid the risk of intrasexual aggression, by avoiding to occupy water pools used by larger males.

Chapter II also provided evidence that male boldness affected their responses to chemical cues of familiar and unfamiliar male turtles. Shy individual male turtles spent more time in the pool with their own chemicals, avoiding pools with chemicals from either familiar or unfamiliar males. In contrast, bold males preferred the chemicals of familiar males to the chemicals of unfamiliar males. Boldness is associated with dominance (Sundstrom et al. 2004; López et al. 2005b; Colléter & Brown 2011) and has been related with winning ability in a context manner during social aggressions (Fuxjager et al. 2010). Hierarchy formation during familiarity process could explain the pattern obtained in Chapter II, probably because turtles may learn to be winners or losers based on their prior experience (Whitehouse 1997). Thus, shy males should be in a previous disadvantageous situation in agonistic encounters and competition for the resources. Otherwise, bold males selected pools with odours of familiar males probably because they could be victorious in agonistic interactions.

Function of sexual dichromatic coloration

Chapter III results showed the existence of chromatic differences between sexes of *M. leprosa* terrapins in the limb stripes but not in the shell (Figure 6). Females had limb stripes that were significantly brighter and with more ultraviolet (UV) saturated coloration than males. Natural selection imposes a strong force that may modulate the various aspects of colour patterns (Endler 1980). In this way, shell coloration may have a cryptic function because is directly exposed to visually-oriented predators (Ross & Lovich 1992; Rowe et al. 2013). Otherwise, turtles can hide inside their shells making

their stripes of the head and legs more difficult to detect by predators. Therefore, limb coloration could be more easily subjected to sexual selection forces than shell coloration.

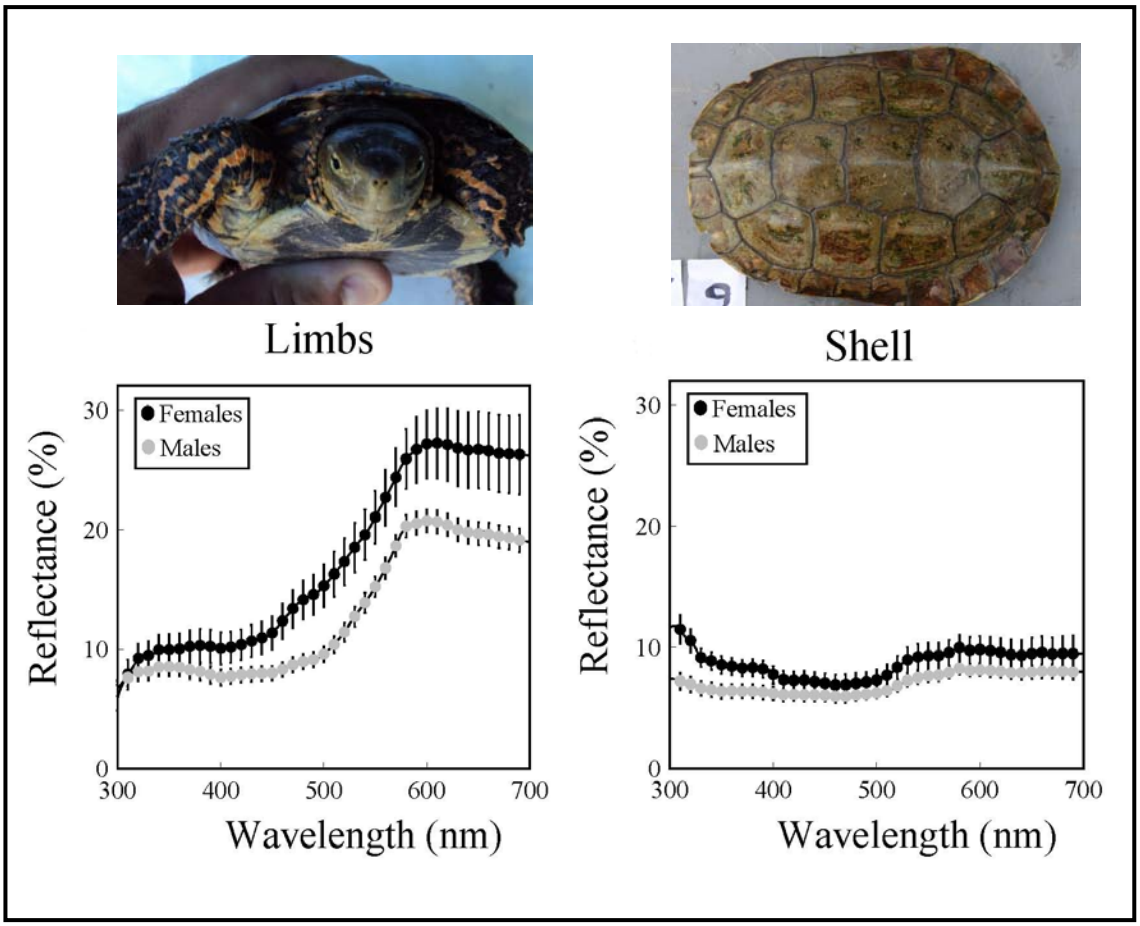

Figure 6. Mean (± SE showed at 5 nm intervals) reflectance spectra of limb coloration (left) and shell coloration (right) of female (black dots) and male (grey dots) M. leprosa *terrapins. Picture credits: Alejandro Ibáñez and José Martín.*

Moreover, characteristics of coloration of the limb stripes were related with body size and health state in females but not in males. Larger females and had more UV saturated limb stripes coloration, and females with better immune responses had brighter limbs stripes. Thus, female ornamentation may reliably signal multiple different aspects of the phenotypic quality of the female or her potential offspring,

allowing males to assess the quality of potential mates in basis of their reproductive colour expression (Weiss 2006; Weiss et al. 2011). Similarly, female *M. leprosa* with brighter limbs might indicate to their potential mates a greater immune response that could be heritable for the future offspring. In addition to visual cues (limb brightness), female *M. leprosa* seem also able to signal their immune response though chemical cues (Ibáñez et al. 2012) in an additive way, increasing linear strength of preferences for possible mates (Kunzler & Bakker 2001).

In contrast, shell coloration did not differ between sexes in *M. leprosa* terrapins, although some individual variations in shell coloration might mainly reflect ontogenetic changes in size-dependent predation risk. Larger males had darker and more UV saturated shell coloration and larger females also had more UV saturated shell coloration. The number of bird predator species is higher in small turtle hatchlings (Janzen et al. 2000). Thus, higher predation pressure on smaller turtles might constrain their shell coloration (i.e. less UV reflectance) to avoid being easily detected by bird predators. In addition, hatchlings and juveniles of some freshwater turtles may modulate the colour intensity of their carapaces making lighter or darker their shells in function of the colour substrate (white or black respectively) and this process is reversible allowing the individuals to converge on the substrate colour after experiencing different environments, thus facilitating crypsis (Rowe et al 2006b, 2009). Thus, brighter shells of smaller males might be cryptic if they occupy a different environment with lighter substrates. Alternatively, in the Hermann tortoise, males with darker eumelanic shells are bolder toward humans and more aggressive during the interactions with other males (Mafli et al. 2011). Therefore, the loss of bright in males' shell might be related with dominance, with larger and duller males signalling their higher aggressiveness.

Sources of inter-individual variation in boldness under risk

Results obtained in the **Chapter IV** demonstrated that turtles were able to modify their antipredatory hiding behaviour in relation to the predation risk level. Thus, appearance and waiting times differed significantly across three different levels of risk (Figure 8). Turtles had significantly shorter appearance times when they were placed in a prone position ("low risk" treatment) than when they were overturned in the "medium risk" and "high risk" treatments. Similarly, when turtles were overturned, they had longer hiding times until their appeared from the shell when risk increased as suggested by a longer time being handled by the predator ("high risk treatment") compared with the "medium risk" treatment. Concerning waiting times, turtles had significantly longer waiting times in the "low risk" than in the "high risk" treatment. Differences between waiting times in the "medium risk" and "high risk" treatments only approached significance, and waiting times in the "low risk" and "medium risk" treatments did not significantly differ (Figure 8; next page). Longer appearance times from the refuge (in this case shell) tend to increase the costs associate to refuge use such as lost of time and thermal costs (Martín & López 1999b). Unlike appearance times, during waiting times turtles may monitor and obtain information about the surroundings assessing whether a predator is still present (Polo et al. 2011). Results obtained in this chapter suggested that turtles shift to behavioural costly tactics (i.e. longer appearance times) under perceived high risk conditions, but that uncertainty about predation risk (in the low risk situation) may also increase costs of refuge use due to the need to monitor the actual risk of the potential predator before deciding to switch to an active antipredatory escape strategy or to resume normal activity.

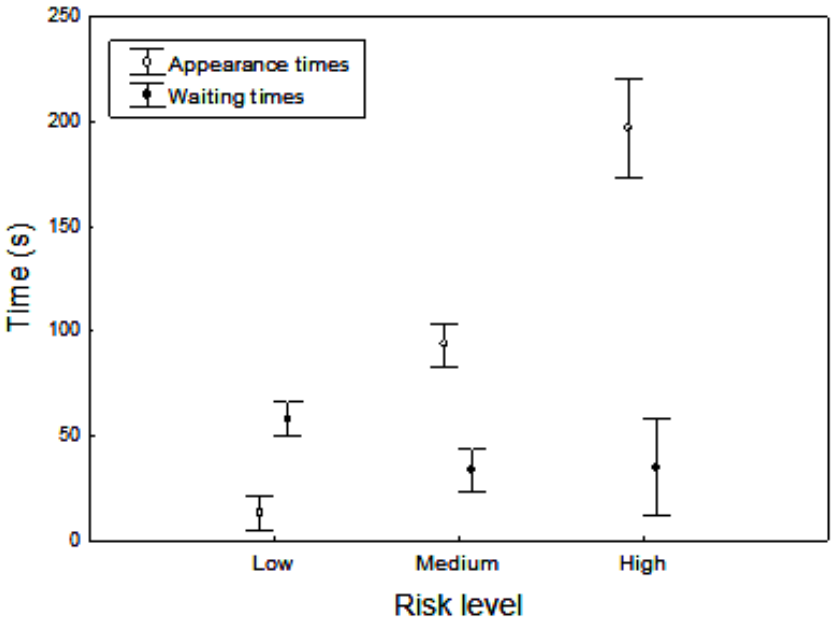

Figure 8. Mean (± SE) appearance and waiting times (s) of the turtles in the "low", "medium" and "high" risk treatments

In addition, boldness under predation risk was affected by several traits, such as gender, body size and coloration. Heavier turtles spent longer times hiding inside their shells after the attack during the "low risk" treatment (prone positioned) and started an active escape earlier than smaller ones in the "medium" risk treatment (overturned). Smaller individuals may emerge earlier from their refuges because they might not afford the thermal (Martín & López 2003) and/or metabolic (Krause et al. 1998) costs of remain inside the shelters. This fact, taken together with a possible greater agility and less detectability to predators might explain why smaller turtles had shorter appearance times. Otherwise, heavier turtles had shorter waiting times when they were overturned. In this way, remaining overturned may entail high costs, such as overheating and/or the loss of feeding and mating opportunities (Ydenberg & Dill 1986; Sih et al. 1990; Martín et al. 2003; Polo-Cavia et al. 2009, 2012). Thereby, the reduced ability of larger turtles to flip over after being overturned, lower predator vulnerability and the costs associated with remaining overturned might favour shorter waiting times in larger turtles.

The gender of the turtle also affected their appearance times during the "medium risk" treatment. Males spent less time than females inside their shells before they

reappeared. Similarly, males of *Testudo horsfieldii* (Bonnet et al. 2001) were more effective and quicker in righting after being overturned than females. In addition, *M. leprosa* females are larger, heavier and their morphological design is more robust than in males (Muñoz & Nicolau 2006). Thus, female *M. leprosa* could be less successful and slower than males in returning to their normal position due to their body size and morphological profile. In this way, appearing later from their shell should increase their chances to escape successfully while the predator remains close.

In addition, brightness of limb stripes was related with appearance times in males, but not in females, and only when risk was low. Males with brighter coloration of limb stripes spent longer times hidden inside their shells in the "low" risk treatment. Limb coloration showed sexual differences, male *M. leprosa* showing less bright and less UV saturated stripes than females (Ibáñez et al. 2013). However, the smaller size of males could render them more vulnerable to predators and showy individuals might be shyer and have longer hiding times due to their higher risk of being detected by predators (Endler & Greenwood 1988; Mänd et al. 2007).

Effect of reproductive state in exploratory activity and boldness

The results of **Chapter V** evidenced that some components of boldness (i.e. appearance times) but not exploratory activity of female turtles were affected by their reproductive state. Thus, gravid turtles behave shier and had significantly longer appearance times from inside their shells than non-gravid females in both the "low" and "high risk" treatments. Otherwise, reproductive state did not affect waiting times although gravid females with more eggs tended (but not significantly) to have longer waiting times in the "low" risk and shorter waiting times in the "high" risk level. Differences in

appearance times between gravid and non-gravid females might be attributed to the trade off between current and future reproductive effort. The current reproductive value of gravid females is much higher than that of non-gravid ones. The "asset-reproductive principle" predicts that the larger an individual's current reproductive value is, the more important it becomes that the asset be protected (Clark 1994). Thus, gravid females invested relatively more in current reproduction than non-gravid ones and should be more cautiously in front of a potential predator attack. Concerning waiting times, gravid turtles with more eggs could need to bask for longer periods as it occurs in gravid lizards (Shine 1980). In this way, gravid females with greater clutch sizes could need longer waiting times (in the "low" risk treatment) to reach an optimal temperature due to the higher requirements of egg production. Paradoxically, females carrying more eggs tended to have shorter waiting times under high risk, probably because in this context the turtle had assumed that had already been detected by the predator rendering unimportant other traits.

In addition, explorative behaviour was not affected by reproductive state of the female turtles. Exploratory behaviour has been shown as heritable and advantageous in some contexts, for example, in great tits faster exploratory parents has offspring with greater ability to disperse in wild habitats (Dingemanse et al. 2003). Similarly, more exploratory lizards in a novel environment are able to enhance fast predation risk assessment abilities (Rodríguez-Prieto et al. 2011). However in our study, gravity did not affect boldness in exploratory behaviour of female turtles, suggesting that this factor might be unimportant for exploration in new environments or that the effect might be masked by other factors.

Lastly, boldness in appearance and waiting times were not related to boldness in exploratory behaviour of female terrapins. In contrast, juvenile convict cichlids

(*Amatitlania nigrofasciata*) that were more exploratory in a novel environment were slower to react to a simulated fish predator attack (Jones & Godin 2010). However, the results in female Spanish terrapins suggest that boldness in response to a predator attack and boldness in exploratory behaviour are independent.

Basking behaviour, health state and colour conspicuity

The results of the **Chapter VI** showed that basking behaviour activity was related with health state and colour showiness of the limb stripes in male Spanish terrapins. Turtles that spent more time basking had lower white blood cells (WBC) counts and lower values of haematocrit. In addition, male turtles with less bright limb stripes had higher basking activity. Otherwise, body size did not influence basking behaviour of the turtles.

Basking activity was related with some variables that can reliable indicate the physiological-health state of the turtles, such as haematocrit and total WBC count. For example, erythrocytes have a pivotal role in tissue oxygenation. An increased haematocrit might be an adaptation for air-breathing vertebrates that remain diving for long periods in aquatic habitats. For example, sea turtles have the highest values of haematocrit in reptiles and are comparables to the values found in marine mammals (Lutcavage & Lutz 1997). Thus, a higher value of haematocrit might provide a higher aerobic capacity improving locomotor performance and mobility in male terrapins, as happens in the lizard *Amphibolurus nuchalis* (Garland & Else 1987). In addition, leukocyte profiles can provide a reliable assessment of stress in vertebrates (reviewed in Davies et al. 2008). In the case of H:L ratio, it may be used as a compliment to the measurement of adrenal hormones in the study of vertebrate stress responses (Davies et

al. 2008). For example, individuals of *E. marmorata* living in a wastewater treatment plant, subjected to a high level of pollution, had higher H:L ratio than those turtles living in an ecological reserve (Polo-Cavia et al. 2010). However, in our study basking activity was not related with the H:L of the turtles. On the other hand, the relation of total WBC counts with individual stress seems inconsistent across the literature (reviewed in Davis et al. 2008). Infections commonly cause general increases in total WBC counts in vertebrates (Davis et al. 2008). For example, chickens that had an acute inflammation experienced a significant increase of leukocytes persistent through seven days (Latimer et al. 1988). However, it is not clear whether higher total WBC counts reflect a better or a worse immune system. For example, offspring of ornamented male three-spined sticklebacks, *Gasterosteus aculeatus,* have higher WBC counts, which are interpreted as a greater parasite resistance inherited from their parents (Barber et al. 2001). In freshwater turtles, both total white and red cells counts have been evaluated together in some studies suggesting their dependence on ecological conditions and individual nutritional state. In freshwater turtles of the species *Emys orbicularis* and *Mauremys rivulata*, blood cell counts depended on habitat quality with an increase of erythrocytes and leukocytes in contaminated water (Tosunoglu 2011). The authors argued that the high leukocyte values are due to water pollution, while high erythrocytes values were caused by decrease in oxygen (Tosunoglu 2011). However, western pond turtles from contaminated habitats had lower immune response than those from non-contaminated habitats but the total WBC counts did not depend on the habitat alteration (Polo-Cavia et al. 2010). In addition, malnourished giant Amazon turtles (*Podocnemis expansa*) had lower values of haematocrit and lower total WBC counts than turtles fed in normal conditions (Tavares-Dias et al. 2009). Thus, a possible explanation for the low haematocrit values observed in the terrapins with high basking activity might be

that they had more static lifestyle and consequently a reduced oxygen demand. Similarly, male terrapins that dedicate more time to basking might reduce the time spent in other activities, such as foraging, compromising the amount of energy invested in their immune system, and consequently have lower total WBC counts. In any case, our results seem to indicate that an increased basking activity might compromise the health state of male turtles.

Different components of coloration may reflect several phenotypic traits in several reptiles (Weis 2006; 2011; Martín and López 2009). Showy coloration in males of several species may be favored through sexual selection (Andersson 1994). In birds, predation risk is likely to influence the evolution of coloration, disfavouring bright coloration in some species (Götmark 1993). In freshwater turtles, shell is the most visible part exposed to predators during basking activity. In fact, shell colour variation across different habitat types seems correlated with substrate ground coloration (Rowe et al. 2006a; McGaugh 2008). Previous studies with different species of freshwater turtles have suggested that shell coloration might be involved in crypsis (Rowe et al. 2013; Ibáñez et al. 2013). Thus, we did not consider shell coloration for our study. Otherwise, the head and limbs of turtles can be hidden, at least partially, by retracting them inside the shell and are not always exposed to predators. Thus, limb coloration can be more easily subjected to sexual selection forces and withstand an exaggerated ornamentation than shell coloration (Ibáñez et al. 2013). Thereby, terrapins having brighter limb stripes might be more conspicuous for predatory birds. In line with those results, males with brighter coloration in their limb stripes behave shier after a simulated predatory attack and have longer hiding times inside their shells (Ibáñez et al. Chapter V). In freshwater turtles, aerial basking in terrestrial habitats may be risky. For example, wood turtles are able to regulate their body temperature through basking

behaviour, but thermoregulation is imprecise suggesting that predation risk could constrain basking behaviour while turtles are exposed on bare ground (Dubois et al. 2009). Thus, turtles having striking coloration (i.e. brighter skin in limb stripes) should assume higher risks during basking activity than those having less striking colours. In this context, more conspicuous individuals may reduce basking times to reduce the probability of being detected and captured by potential predators.

Conclusions

1. Spanish terrapins are able to communicate via chemical signals in water released by conspecifics. Females probably have a health condition-dependent chemical signal informing about their immunological response that affect responses of males. This would suggest that male turtles may base their mating preferences on chemical cues that honestly report the female's health state and, thus, invest in the quality of their future offspring. Otherwise, chemicals of males may convey information about their size to females and other males. Probably, male *M. leprosa* of different sizes may differ in the chemical composition of their gland secretions, or in the amount of chemicals secreted. Female choice of pools occupied by larger males could protect them from the harassment and forced inseminations of other males. On the other hand, in males, chemical detection and avoidance of chemicals of larger and stronger males may prevent agonistic encounters with these males, in which smaller males could be at a disadvantage, allowing them to avoid injuries and save energy. In addition, male Spanish terrapins are able to distinguish between familiar and unfamiliar chemical cues found in the water. Similarly, male-male interactions in the Spanish terrapin could depend on the individual personality along the shy-bold axis and consequently affect their responses to conspecific chemicals. Bold males occupy pools with chemicals from familiar males unlike shy males that avoid familiar chemical cues. Nevertheless, both shy and bold males avoid to occupy pools containing odours of unfamiliar males. Taken together, these results suggest that male turtles might establish hierarchies during familiarization process, with bold males being dominant over shy ones. On the other hand, chemical cues from unfamiliar males might represent a hazard for both shy and

bold males. Thus, boldness and body size could influence intrasexual competition between males.

2. Spanish terrapins show sexual dichromatism in limb but not in shell coloration. Males have less bright limb stripes than females suggesting that coloration is more important in sexual selection for females. Moreover, limb coloration of females seems to have higher variability than in males, and individual variation is related with size and health state, which suggests that female coloration may be implicated in mate choice. Sexual selection forces could act strongly on female limb coloration promoting honest signals that are reliable for their receivers. Alternatively, duller coloration of limb strips in males might be linked to melanic changes related with age or size. In addition, the small size together with the elevated mobility of male terrapins might carry on a higher predation risk and natural selection should favour less bright coloration. In contrast, shell coloration does not differ between sexes, although it is related with body size in males and females. Smaller turtles have smaller amount of UV reflectance in their shells, which could render small turtles more difficult to detect by bird predators suggesting that shell coloration might have a cryptic function.

3. Individuals of *M. leprosa* are able to assess the predation risk level in their environment and optimise their antipredator hiding response. Appearance times increase in concordance with the risk perceived by turtles, which behave more cautiously when perceived predation risk increases. However, waiting times decrease when risk increases, suggesting that turtles need more time to assess and monitor whether a predator is still present when the uncertainty of future risk is greater. The turtles show a size-dependent response, with longer appearance times for larger turtles when they are prone. For males, limb brightness is the main factor affecting their appearance times, but only in the “low” risk treatment. Nevertheless, appearance times when turtles are

overturned are not affected by size, but males re-emerge from their shells relatively sooner than females. Waiting times are affected by size in the “medium” risk treatment, with smaller turtles starting to right later than larger ones. Taken together, these results suggest that males and females differ in terms of the traits that influence their boldness in risky contexts in different ways and that different aspects of the antipredator behaviour might be influenced by different factors. Similarly, conspicuousness to predator eyes (i.e. larger size and brighter colours) might lead to differences in appearance times in terrapins. Nevertheless, while the risk level increases, the turtles spend longer times inside their shells and need less time to initiate an active escape, and the relative contribution of size and coloration is less important (i.e. no relation in the “high” risk treatment). Thus, when risk increases, a turtle may assume that it has already been detected by the predator and then traits like body size or the degree of conspicuousness should not be important for their boldness in hiding behaviour, making the inter-individual differences in those traits irrelevant.

4. Hiding and exploratory behaviour are not related providing evidence that both traits are not a behavioural syndrome in female Spanish terrapins. The results obtained highlight that under risky situations, some components of boldness (i.e. appearance times) are affected by reproductive state of females. However, exploratory behaviour or waiting times do not differ between reproductive state classes. Gravid females appear relatively later from into their shells after a predatory attack than non-gravid ones. In addition, gravid females carrying a greater number of eggs tend, although not significantly, to have longer appearance times as well as longer waiting times under “low” risk but shorter waiting times under “high” risk. Those results may suggest that larger clutches could affect boldness in risky contexts of Spanish terrapin females. The lack of differences in relative and absolute weight between gravid and non-gravid

females suggest that differences in appearance times might be due to metabolic-physiological costs and a worse condition of gravid females associated with egg production and embryo maintenance. However, gravid females might act more cautiously to protect their immediate higher fitness with respect to non-gravid ones.

5. Basking activity was related with health state (i.e. total number of leukocytes and haematocrit) and limb coloration. Male turtles that spent less time basking had higher total WBC counts and higher haematocrit values. Thus, males with higher basking activity could have less oxygen requirements due to their lower mobility and consequently reduced metabolical expenditure. Similarly, males that spent more time basking could be more susceptible to infection due to the fact that they dedicate less time to foraging which may compromise their immune system. Otherwise, basking behaviour could carry associate costs in terms of predation risk with more conspicuous individuals reducing their basking activity to avoid be easily detected by potential predators. Further studies should include females to clarify the role of coloration showiness in basking patterns in freshwater turtles.

Future perspectives

The results of the present thesis answered several interesting questions about the ecology and behaviour of Spanish terrapins. However, this study raises new points which could be addressed at a later stage of research considering further perspectives.

As evidence of intra- and intersexual chemical communication in *M. leprosa* was provided here, it would be interesting to disentangle the question of whether bigger males secrete more amounts of chemicals to the water or, whether large and small males differ in their profile of chemical compounds. In the same way, the result that females may signal their immunological state with chemical compounds opens a door to investigate whether pheromone signalling is condition dependent, where perhaps females with a higher immunocompetence may release different compounds or more amounts of them. One option would be to plan an experiment where every turtle might try the smell of its conspecifics diluted in different proportions. Thus, for example a turtle might be exposed to diluted and undiluted odour of one male. In this context, the responses of the turtles to the diluted versus undiluted odour of males of different size would give us an idea of whether the releasing of pheromone is size-dependent in males. In addition, the determination of the chemical profile of the gland secretions in this species would be essential to understand which substances play a pivotal role in mate choice and intra-sexual interactions.

One approach that may help to understand the processes underlying coloration, and sexual selection would be conducting experiments. For example, one interesting option would be to use artificial models imitating real female turtles, painted in a range of colours from darker to brighter. However, for a more realistic approximation it could be also used females of similar characteristics (i.e. same size and age) differing in a

range of coloration or with coloration experimentally manipulated. Thus, one interesting experiment would be one where male turtles could choose between those females or models only using visual cues. Previous results suggest that male terrapins should be attracted to those females having brighter limb stripes. This approach would make it possible to know if coloration of the limb stripes is implicated in mate choice, strengthen the hypothesis that bright coloration has evolved as an honest signal in female Spanish terrapins.

Results found in the last chapter showed that male terrapins with higher basking activity had lower leukocyte and haematocrit values suggesting that they have a worse health state. These results are not in line with the "desiccation hypothesis" that predicts that aerial basking is the proximate cause to encourage to leeches detaching from the basking turtles to avoid desiccation. Thus, a first step should be to explore the relation between parasite load and basking patterns in wild populations of freshwater turtles. Especially interesting would be to examine whether those males that spend more time basking also have higher parasite load. Mostly of the research on freshwater turtle parasites have mainly focused on aquatic ecosystems. One idea to explore could be whether the vectors involved in the transmission of parasites infecting turtles live in terrestrial habitats. In this context, turtles that spend more time out of the water basking might be subjected to a higher infection rate by vectors of parasites such as mosquitoes. Thus, additional studies on this topic would provide a better characterization and understanding of the ecology of the parasites and their vectors in terrestrial habitats and the effects on turtle behaviour.

Another insight that should be tested is the effect of coloration and reproductive state on basking activity in females. We found that female turtles have limb stripes with brighter and more UV saturated coloration than males. Therefore, females should be

more conspicuous than males for predator eye. Otherwise, we also found that antipredator hiding behaviour depended on limb coloration in males but not in females of *M. leprosa*. Probably female turtles have a lower predation risk than males due to their larger size that would make them more difficult to capture by local predators. Thus, one interesting hypothesis to test would be whether basking activity of female turtles is not dependent of their coloration. Otherwise, reproductive state of females could modulate their basking behaviour. In one hand, gravid females should bask during longer periods of time than non-gravid ones because their higher thermoregulatory requirements for embryo development. On the other hand, as we showed in this thesis, gravid females should behave more cautiously after a predator attack than non-gravid ones. In the same way, females carrying clutches should also be more carefully during basking activity due to their higher reproductive value. This, would support the hypothesis of behavioural compensation for gravid female turtles also in the field. Thus, monitoring basking activity of both gravid and non-gravid turtles would provide a better point of view of the possible costs derived of reproduction in freshwater turtles.

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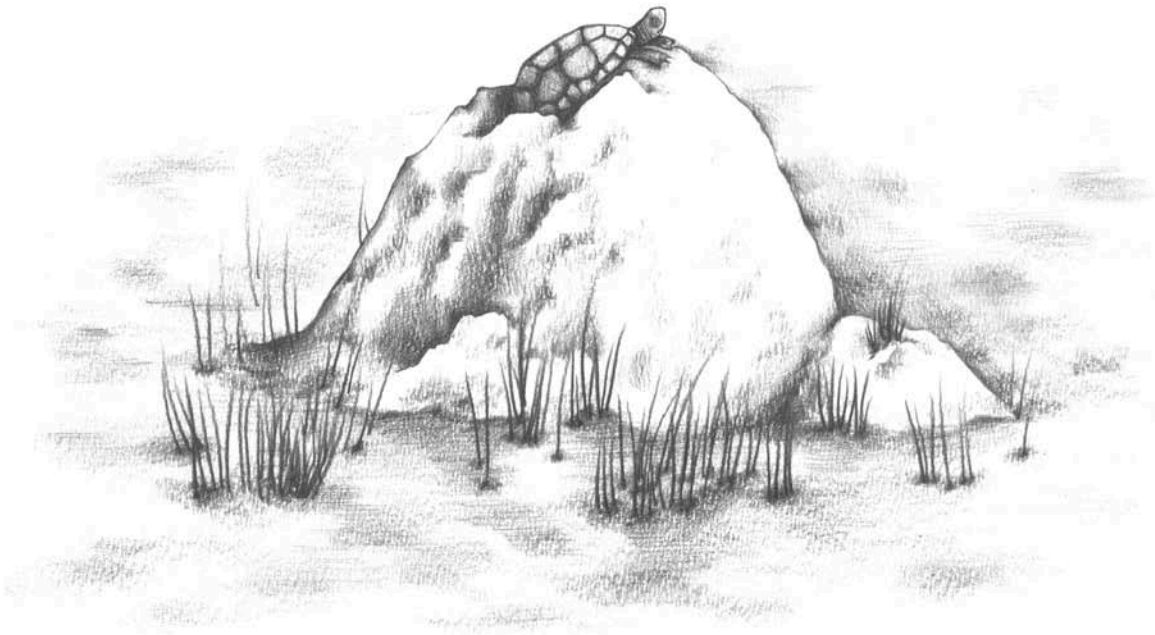
Chapter I



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Discrimination of conspecifics' chemicals may allow Spanish terrapins to find better partners and to avoid competitors

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Abstract: Chemosensory discrimination of sex and other individual traits based on pheromones occurs in many reptiles. However, the use of chemoreception in social behaviour of turtles has been little studied. We examined the role of chemical signals in intraspecific communication of Spanish terrapins (*Mauremys leprosa*) during the breeding season. Chemical cues released by terrapins in the water might inform on sex and other individual characteristics, such as size, body condition or health state, which are important in sexual selection processes. To test this hypothesis, we compared the time spent by turtles in water pools with chemical stimuli obtained from different conspecifics. Behavioural responses showed that turtles were able to detect and discriminate chemical cues released by conspecifics, and to modify their use of space in response to these cues alone. Male turtles preferred water pools with chemicals of females with greater immune responses, while females preferred water pools with odours of larger males. Preferences for water used by better potential partners could increase mating opportunities with these partners, which would increase offspring fitness. Nevertheless, female preference of large males could also be a mechanism of protection from harassment of small males. On the other hand, males avoided water pools with chemicals of heavier, or relatively heavier, males and preferred water with chemicals of smaller, or relatively smaller, males. Chemosensory assessment of body size may allow males to minimise the risk of costly aggressive interactions with other males. Selection of water pools based on chemical cues of conspecifics may allow terrapins to optimise their mate searching while avoiding competitors.

Keywords: chemical communication, sexual selection, pheromones, mate choice, male competition, body size, health state.

Introduction

Numerous studies have shown the importance of chemical signals in social and sexual behaviour of many animals (Wyatt 2003; Mason & Parker 2010). In reptiles, intraspecific chemical communication has an important role in mate searching and mate selection, as well as in discrimination of the quality of potential partners (Cooper & Vitt 1984, 1986; Mason 1992; Martín & López 2000, 2011; Mason & Parker 2010). For example, in some lizard species, females may choose males based on the odours emitted by femoral glands of males, which may contain specific information about males' traits (Olsson et al. 2003; López & Martín 2005; Martín & López 2006, 2011). Chemical signals are also used in intrasexual relationships between males, where they can contribute to reduce costs of aggressive interactions (Carazo et al. 2007; Martín & López 2007; López & Martín 2011).

Studies on chemosensory abilities of turtles have received little attention compared to other groups of reptiles such as lizards and snakes (reviewed in Mason & Parker 2010; Martín & López 2011). However, many studies have focalized on the nasal and vomeronasal system, showing that these are well developed in turtles (Halpern 1992; Hatanaka & Matsuzaki 1993; Fadool et al. 2001), which suggests an ability of turtles to detect chemical cues in the environment. For example, Painted turtles (*Chrysemys picta*) are able to discriminate chemicals in the water of the pond where they live versus the water of new ponds, but only females prefer to occupy the water of their original ponds (Quinn & Graves 1998). More recently, laboratory experiments showed the importance of inter- and intrasexual chemical communication in turtles (Muñoz 2004; Poschadel et al. 2006; Galeotti et al. 2007; Lewis et al. 2007; Polo-Cavia et al. 2009). Spanish terrapins (*Mauremys leprosa*) are able to detect odours

of conspecifics and avoid water pools with chemicals from the competitor exotic terrapins *Trachemys scripta* (Polo-Cavia et al. 2009). In addition, outside of the breeding season, male and female *M. leprosa* terrapins avoid to occupy water with chemicals of conspecifics of the opposite sex (Muñoz 2004). On the other hand, during the breeding season males choose water with chemical cues of females, and females prefer water that contains chemicals from other females (Muñoz 2004).

In this paper, we analysed the behavioural responses of Spanish terrapins (*M. leprosa*) to chemical cues of conspecific individuals of different body size and health state. In a first experiment, we examined the potential role of chemical cues in partner choice. We performed a laboratory experiment where each turtle could choose between a pool with clean water and a pool with water with chemical stimuli from individuals of the opposite sex. We wondered if turtles were able to discriminate some individual traits that may be important in mate choice, such as body size or health state, through chemical cues alone emitted by conspecifics of the opposite sex. Selection of larger males as potential partners has been reported in several species of lizards (Cooper & Vitt 1993; Wikelski et al. 1996; Censky 1997; Martin & Forsman 1999). Females may choose larger males to obtain offspring of higher genetic quality (Cooper & Vitt 1993). Alternatively, the high degree of harassment and forced inseminations in turtles (Berry & Shine 1980) suggests that females may obtain benefits through mate-guarding by larger males, allowing more time for foraging (Censky 1997) or it may be a form of damage control in response to male harassment (Lee & Hays 2004). In the same way, male mate choice of larger females could be explained because bigger females may contain a greater number of eggs in their abdominal cavity, producing larger clutches and increasing fecundity (Berry & Shine 1980; Wallis et al. 1999; Zuffi et al. 1999; Poschadel et al. 2006). On the other hand, costs of maintaining the immune system

function may create a trade-off between the immune system and sexually selected characters (Wedekind & Folstad 1994). Thus, only individuals with good condition could maintain a great immune response and extravagant secondary sexual traits (Sheldon & Verhulst 1996; Westneat & Birkhead 1998). Pheromones may provide honest indicators of bacterial infection and allow assessment of the health state of potential mates (Penn & Potts 1998; Zala et al. 2004; López & Martín 2005). We predicted that chemical cues emitted by turtles may honestly signal body size and health state. Thus, turtles should choose the odours of larger and healthy mates, but this choice may depend on the relative importance of size and health state of potential partners in each gender.

In a second experiment, we examined the behavioural responses of males to chemical cues of other males. We performed an experiment where each male could choose between a pool with clean water and a pool with water containing chemical stimuli from other males. A large body size could be advantageous in situations of competition for resources, such as obtaining optimal basking sites (Cadi & Joly 2003; Polo-Cavia et al. 2010a). In juveniles of the turtle *Chelydra serpentina* stable hierarchies are formed during competition for food, and body size is the main factor that influence the position occupied in the hierarchy (Froese & Burghardt 1974). In the European pond turtle (*Emys orbicularis*), smaller males tend to avoid the smell of larger males, which are theoretically physically stronger, while larger males tend to choose odours of smaller males, which are theoretically inferior physically (Poschadel et al. 2006). We hypothesised that size and relative size of male pairs should be the main and most important factor in agonistic interactions between males. In this case, males should avoid to occupy water with chemical cues of larger males to reduce the risk of intrasexual aggression, and should prefer to occupy water with chemical stimuli of

smaller males, which may be successfully attacked to increase the probability of mating with females living in the area.

Material and methods

Study Animals

The Spanish terrapin (*M. leprosa*) is a medium sized semi-aquatic species, which is found in Southwestern Europe and Northwestern Africa (Busack & Ernst 1980; Da Silva 2002). These turtles preferably inhabit ponds and streams with riparian vegetation, being less common in large rivers and reservoirs. Individual turtles can be easily sexed based on plastron shape and preanal tail length; males have concave plastrons and greater preanal tail lengths, whereas females can be identified by their flat plastrons and small preanal tail lengths (Pérez et al. 1979; Meek 1987). Males are sexually mature at seven years old, with a carapace length of about 12-14 cm, while females acquire sexual maturity at an age of ten years old, with a length of about 14-16 cm (Pérez et al. 1979). The breeding season occurs in spring, and females can lay between one and 13 eggs (Andreu & López-Jurado 1998; Arnold & Ovenden 2002).

During March of two consecutive years (2010 and 2011), just before the beginning of the mating season (Andreu & López-Jurado 1998), we captured a total of 61 adult turtles (33 males and 28 females) in several ponds, small streams and tributaries of the Guadiana River. These freshwater habitats, located in a dehesa-oak woodland close to Olivenza and Alconchel (Badajoz province, SW Spain), contain a large population of turtles. We used a modified version of the traditional underwater funnel traps, which include a mesh chimney that reaches from the body of the trap to the

surface allowing the turtles to come to the surface to breath (Kuchling 2003; T & L Netmaking, Mooroolbark, Victoria, Australia). We baited traps with sardines and protected them from terrestrial predators by waiting in the proximity. We revised the traps every hour to collect turtles. All collected turtles were alive and did not show any sign of being stressed inside the traps.

Turtles were transported by car in plastic containers with some water, and housed at "El Ventorrillo" Field Station, near Navacerrada (Madrid province) where experiments were conducted. Turtles were housed individually in outdoor plastic aquaria (60 x 40 cm and 30 cm height) containing water and rocks that allow turtles to bask out of water. The photoperiod and temperature were the same as those of the surrounding area. Turtles were fed three times a week minced meat, earthworms, slugs, and a commercial compound of fish "pellets". Turtles were held in captivity for more than two weeks before starting the experiments to allow acclimation to captivity conditions. We performed all the experiments during spring coinciding with the mating season of this (Andreu & López-Jurado 1998) and most temperate-zone turtles (Moll 1979). All individuals were healthy and in good condition during the tests, and at the end of the experiments, all of them had maintained or increased their body mass. Turtles were then returned to the exact locations of capture and released. Captures and experiments complied with all current laws of Spain and the Environmental Agency ("Consejería de Industria, Energía y Medio Ambiente") of the "Junta de Extremadura" Local Government (permit numbers: CN10/59 and CN0008/11/ACA). Procedures were conformed to recommended guidelines for use of live Reptiles in laboratory research (ASIH 2004).

Estimates of Size and Health

We used a metal ruler to measure to the nearest millimetre the body size (shell) of turtles: maximum carapace length was measured as the greatest straight-line distance from the anterior end to the posterior end of the shell (females: $\overline{X} \pm 1$ SE = 182 ± 4 mm, range = 140-224 mm, N = 28; males: $\overline{X} \pm 1$ SE = 164 ± 3 mm, range = 128-197 mm, N = 33). We used an electronic balance (precision: ± 1 g) to measure body weight (females: $\overline{X} \pm 1$ SE = 879 ± 61 g, range = 370-1550 g; males: $\overline{X} \pm 1$ SE = 535 ± 25 g; range = 210-810 g). In addition, we calculated the residuals of the regression between weight and body length (both log-transformed) as a measure of body condition of individuals (Polo-Cavia et al. 2010b).

To assess the immune response of turtles we used the phytohaemagglutinin test (PHA) (Smits et al. 1999; Belliure et al. 2004; Tella et al. 2008). This is a test "in vivo" that estimates the delayed hypersensitivity immune response mediated by T lymphocytes (CMI), but that may also involve components of innate and adaptive immune system (Martin et al. 2006; Tella et al. 2008). Nevertheless, we used this index because it is a standard measure of immunocompetence and health state, regardless of the type of cells involved in it (Polo-Cavia et al. 2010b). We used a spessimeter (accuracy of 0.01 mm) to measure the thickness at the same marked point of the left hind paw of turtles before and 24 h after injection of 0.04 mg of PHA dissolved in 0.02 ml of phosphate buffered saline (PBS). General health of turtles was not affected by this test. The only noticeable effect of the injection of PHA was a slight swelling of the skin that disappears after 48 h. We calculated the immune response (CMI) as the difference between the thickness of the leg at the injection site before and after antigen injection (Belliure et al. 2004; Martin et al. 2006; Polo-Cavia et al. 2010b). We also calculated

the residuals of the regression between immune response and weight (both log-transformed) to remove the possible effects of body size on immune response.

Experimental Design

We aimed to analyse whether *M. leprosa* turtles are able to estimate individual characteristics of conspecifics through chemical cues present in water. For this, we compared the time that turtles spent in a pool with clean water versus the time spent in a pool with chemical stimuli. We used this design rather than a two-choice test with two individual stimuli presented simultaneously because responses to one individual stimulus would not be independent but strongly dependent on the simultaneous response to the other individual stimulus (see Martin & Bateson 1986), which will confound the interpretation of preference indexes. The chemical stimuli came from different individual males or females of different body size and health state (see below).

The experiments were conducted outdoor using two artificial pools (two plastic containers of 60 x 40 cm and 15 cm height) connected by stone ramps that allowed turtles to move easily from one pool to another and to bask out of water. In each trial, one of the pools contained clean water and the other was filled with water containing chemical stimuli of one conspecific turtle. Clean water was obtained from a nearby mountain spring stream that did not contain turtles. Water with different chemical stimuli of turtles came from the individual containers where turtles were kept separately. To prepare the chemical stimuli we filled the individual home containers with clean water (18 L) and kept each turtle individually in its container during three days to impregnate the clean water with the scent of each turtle. After this time we used the water with chemical stimuli immediately in the tests to ensure that the chemicals

were not degraded or lost before the trials. To ensure that water contained only chemical secretions of each individual turtle, we did not feed the turtles during these three days, thus avoiding contamination of water with the scent of food items. This is the average time needed for digestion in many freshwater turtles (Mahmoud 1968). Similar starvation periods have been used in other studies without noting adverse effects for turtles (Muñoz 2004; Poschadel et al. 2006; Polo-Cavia et al. 2009). Also, in other freshwater turtles, much longer deprivation periods did not produce weight loss or changes in survival (Belkin 1965). In our study, the three days of starvation had no negative impact over the health of turtles, which had increased their body mass at the end of the study.

We used a total of 33 males and 28 females for obtaining two or three chemical stimuli of each turtle, which were subsequently presented to different focal turtles of the opposite sex. Each focal male (N = 29) or female (N = 30) turtle participated in three trials with chemical stimuli of three different females or males respectively. In the case of the male-male trials, we used a total of 16 males for obtaining two or three chemical stimuli subsequently presented to different males. Each focal male (N = 16) participated in three trials with chemical stimuli of three different males. To avoid that a possible effect of a previous experience in the experimental pools could influence the results, all the turtles participated in the same number of trials. Final sample sizes were smaller because we excluded the trials were the turtles spent less than 10% of the time in one of the two pools.

Tests were only made on sunny days when turtles were fully active, excluding cloudy and rainy days. The positions of the pools (clean *vs.* stimuli), the order of participation in the trials of focal turtles, and the chemical stimuli tested (i.e. the donor turtle) were randomized. We monitored the temperature of pools at the beginning of the

experiments (with a digital thermometer; precision: ± 0.1 °C), the illumination intensity and depth of the water column, to ensure that conditions were similar in both pools during all trials (DeRosa & Taylor 1980). After each test, all pools and ramps were cleaned with clean water and allowed to dry outdoor to avoid contamination with other smells. The experiments were conducted during spring (March to May), coinciding with the breeding season of *M. leprosa* (Andreu & López-Jurado 1998).

Before starting each trial, focal turtles were allowed to bask for at least 2 h in their home containers. At the beginning of each test, the turtles were gently placed in the middle of the stone ramps linking the two experimental pools, to avoid any side-bias in the choice of pools. Turtles were usually very active, shifting between pools, more frequently at the beginning of the experiment and less frequently as time passed by. We used the instant registration procedure (Martin & Bateson 1986), monitoring each turtle from a hidden position for 2 h, and noting the location of the individual every 5 min (24 total records for each turtle in each test). The locations observed on each of the 24 scans were considered to be representative of turtles' space use of the pools because preliminary tests showed that turtles had limited movement rates, spending long time periods stationary, and changed between pools in few occasions. In each record, if the turtle was inside one of the two pools (clean or chemical stimuli), it was designated as having chosen temporarily that particular pool, whereas if the position of the turtle was not clear (e.g., on the ramp), it was designated as having made no choice. The experimenter that made observations was blind respect to the position of the stimuli and the characteristics of the donor turtle. The trials were spaced in time at least two days, none individual participated in one experiment on two successive days to avoid the possible stressful effects on the next test.

We evaluated the "preferences" of each turtle for each chemical stimulus as the proportion of time (excluding time in the "no-choice" places) that each turtle spent in each pool (clean *vs.* chemical stimulus). Preliminary experiments showed no significant differences in time spent by turtles in two pools with clean water.

Data Analyses

Data (weight, length and immune response) were logarithmically transformed to normalize them before analysis (verified with Kolmogorov-Smirnov test; $P > 0.05$ in all cases). We also calculated the arcsine square root of the proportion of time spent in the pool with water with chemical stimuli (Zar 1984). We constructed general linear mixed models (GLMM) with time spent in the pool with water containing chemical stimuli ('preference') as the dependent variable. Size, body condition and health state were included as covariates, and the year was included as a fixed factor to correct for possible time bias. To correct for non-independence of data points, we considered the identity of the turtle acting as the donor of the chemical stimuli as a random effect. Simultaneously, we ran other GLMM (only in the trial of males responding to chemicals of other males) with the difference between the characteristics (size, body condition and immune response) of the focal male and the male donor of the chemical stimuli as covariates. To correct for non-independence of data points, we considered both donor male identity and focal male identity as random effects. To best illustrate the effect due to the variable of interest, we graphically represented our results using a regression analyses between the dependent variable and the variable of interest. We also ran backward stepwise general regression models (GRM) with the average value of each treatment responses (time spent on the water side with chemical stimuli) as dependent variables and size,

body condition and health state as independent variables. These models revealed qualitatively similar patterns to the GLMM (data not shown). All the statistical analyses were performed with STATISTICA v8.0 (Statsoft, Inc, Tulsa, OK, USA).

Results

Responses of Males to Females' Chemical Stimuli

The trials (N = 64) showed that male turtles spent significantly more time in pools with water that contained chemical cues of females with a better health, as estimated from the females' greater immune response (R^2 = 0.34; Table 1; Fig. 1). This relationship was maintained in the two years of the study (i.e. the interaction year*CMI response was not significant). On the other hand, body size and body condition of the female donor of the chemical cues did not significantly affect pool selection (Table 1).

Responses of Females to Males' Chemical Stimuli

In trials of females' response to male's chemicals (N = 70), females spent significantly more time in pools with water that contained chemical cues of heavier males (R^2 = 0.27; Table 2; Fig. 2). The pattern was the same in the two years of the study (i.e. the interaction year*weight was not significant). In contrast, immune response and body condition of males did not significantly affect females' choice (Table 2).

Table 1. Relationship between the time spent by male turtles in water pools with chemical cues of conspecific females and the individual characteristics of female donor of those chemical stimuli. Significant effects (P < 0.05) are indicated in bold.

	Time spent in water with chemical cues	
Covariates	$F_{1,35}$	*P*
Year	0.12	0.73
Weight	0.40	0.54
CMI response	**6.12**	**0.03**
Body condition	0.89	0.36
Year*Weight	1.09	0.31
Year*CMI response	0.01	0.97
Year*Body condition	0.15	0.70

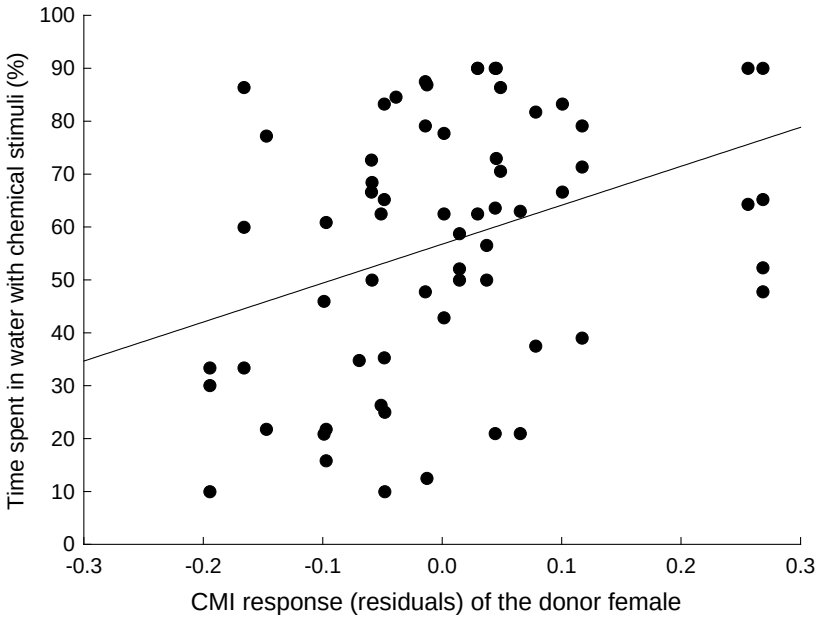

Figure 1. Behavioural preferences of male turtles for the chemicals of conspecific females with different health state (i.e. the residuals of the cell-mediated immune response, CMI, on weight) during the breeding season. Data points represent each trial. 'Preferences' were estimated from the percentage of time spent in the water pool that contained chemical stimuli of a given female (see methods).

Table 2. Relationship between the time spent by female turtles in water pools with chemical cues of conspecific males and the individual characteristics of males acting as the donor of those chemical stimuli. Significant effects (P < 0.05) are indicated in bold.

	Time spent in water with chemical cues	
Covariates	$F_{1,37}$	*P*
Weight	**6.88**	**0.017**
CMI response	0.05	0.82
Body condition	0.14	0.71
Year	1.18	0.29
Year*Weight	0.29	0.59
Year*CMI response	0.01	0.91
Year*Body condition	0.03	0.87

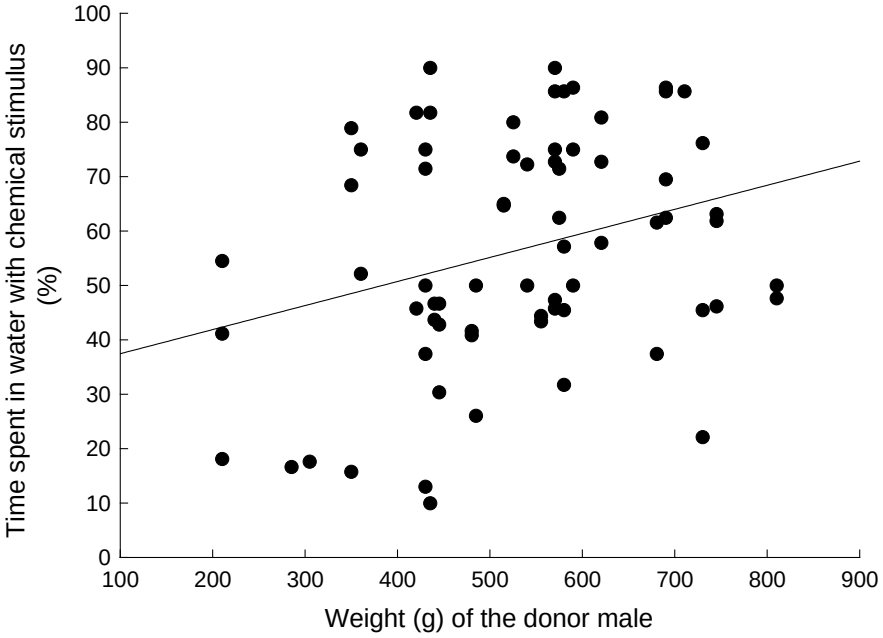

Figure 2. Behavioural preferences of female turtles for the chemicals of conspecific males of different body size (weight, g) during the breeding season. Data points represent each trial. 'Preferences' were estimated from the percentage of time spent in the water pool that contained chemical stimuli of a given male (see methods).

Responses of Males to Males' Chemical Stimuli

In the trials testing responses of males to males' chemicals (N = 42), we found a significant effect between the time spent in pools with water with chemical stimuli and the weight of the male that provided the chemical stimuli ($F_{1,26}$ = 16.94, P = 0.0027, R^2 = 0.66; Fig. 3a). Thus, males avoided to occupy pools with chemical stimuli of heavier males. However, there were not significant effects of body condition ($F_{1,26}$ = 2.47, P = 0.14) or immune response of the donor male ($F_{1,26}$ = 0.0002, P = 0.98).

We found similar results when we ran a GLMM with the differences between the characteristics of the focal male minus those of the donor male (N = 42). There was a significant effect between the time spent in water with chemical stimuli and the male relative weight ($F_{1,15}$ = 9.09, P = 0.007, R^2 = 0.33; Fig. 3b), whereas there were not significant effects of the relative body condition ($F_{1,15}$ = 2.81, P = 0.10) or the relative immune response ($F_{1,15}$ = 0.71, P = 0.41).

Discussion

The results obtained in our study suggest that Spanish terrapins (*M. leprosa*) are able to recognise some individual traits of conspecifics through chemical cues alone, and that turtles can modify their behaviour and use of space (i.e. choice of water pools) in response to these chemical stimuli.

Female *M. leprosa* preferred water with chemicals of heavier males, but the body condition and immune response of the male did not appear to be important. In reptiles, female mate choice based on males' traits has been rarely reported (Olsson &

Madsen 1995, but see a revision of recent evidences in Mason & Parker 2010; Martín & López 2011). Female mate choice seems more common in aquatic turtles than among

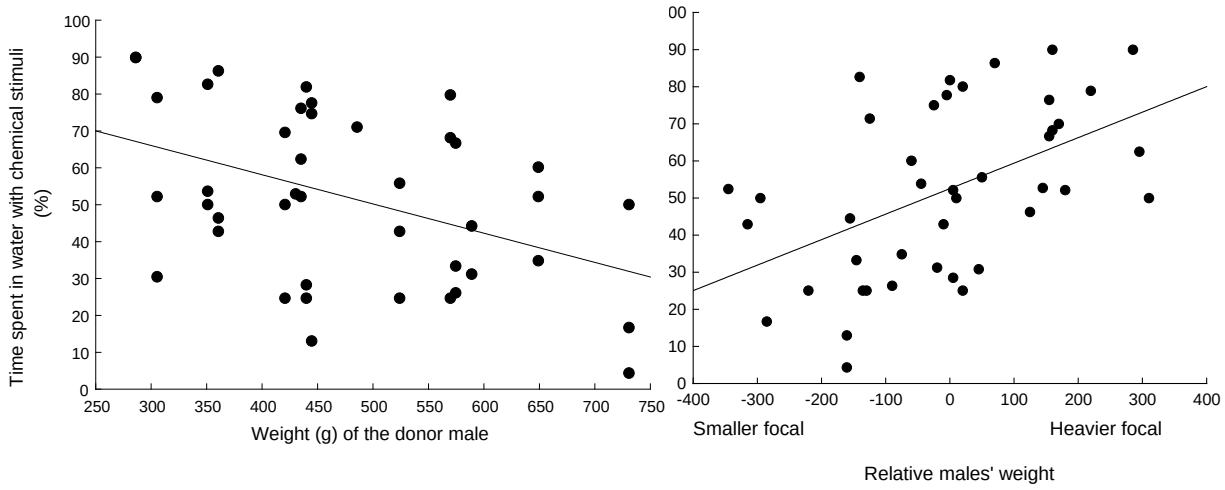

Figure 3. Behavioural preferences of male turtles for the chemicals of conspecific males with different (figure on the left) size (weight, g) and (figure on the right) relative size (i.e. the difference between weight of the focal male minus weight of the male donor of the chemical stimuli) during the breeding season. Data points represent each trial. 'Preferences' were estimated from the percentage of time spent in the water pool that contained chemical stimuli of a given male (see methods).

other groups of turtles, it clearly plays an important role in emydid turtles (Berry & Shine 1980) and it has been also reported in *M. leprosa* (Loveridge & Williams 1957), but the nature of the process remains unknown. However, a study with European pond terrapins, *E. orbicularis*, did not find evidence that females choose larger males based on chemicals in water (Poschadel et al. 2006). The authors argued that this is explained because only males actively search for females, although this does not exclude the possibility that females were able to recognise chemical cues of males (Poschadel et al. 2006). In fact, sample size in that study was very low (only eight females) and results were not clear. Other study with *M. leprosa* did not really test female mate choice, but only the general preferences of females for water with chemicals of males (Muñoz

2004). However, in that study, female *M. leprosa* did not select nor prefer water with chemicals of males during the mating season (Muñoz 2004), which could be expected if females preferred some individual males but not others (something that was not tested in that study).

In the turtle *Clemmys insculpta* larger males have a higher number of matings than smaller ones (Kaufmann 1992). This suggests an advantage of larger males with respect to smaller ones, perhaps in forced inseminations, as this seems the predominant mating strategy in freshwater turtles (Berry & Shine 1980). In this way, greater mobility and large home ranges of females during the mating season (Pettit et al. 1995) could reflect a strategy where females' selection of pools occupied by larger males could protect them from the harassment and forced inseminations of other males. Thus, this strategy could allow females more time for foraging, thereby increasing their lifetime reproductive success (Censky 1997). Our results showed that females were able to recognise and choose water pools that had been occupied by larger males using chemical signals alone. Similar results have been found in some lizards (López et al. 2003) and beetles (Trumbo & Eggert 1994; Beeler et al. 2002). However, female turtles did not spend more time in water with stimuli of males with a better immune response. This contrasts with results found in other animal species such as beetles (Rantala et al. 2002, 2003), mice (Zala et al. 2004) or lizards (López & Martín 2005, 2006; Martín et al. 2007a) where chemical signals can honestly report on the quality of the male immune system. In our study, female turtles did not seem able to discriminate the health state (in terms of immunocompetence) of males. An alternative explanation could be that other factors, such as body size, could have more importance in partner choice masking the potential effects of other variables. Therefore, the results obtained in this study suggest that female *M. leprosa*, far from being passive subjects, could play an

active role, searching and selecting their potential partners, being able to discriminate at least the size through chemical cues released by males.

With respect to the response of male *M. leprosa* to water with chemical stimuli of different females, we found a male preference for water with chemicals of healthy females, but body size and condition of females were not important. These results do not match those found in *E. orbicularis*, where males prefer odours from large females (Poschadel et al. 2006). Larger females may have higher fecundity because they have more space to house the eggs inside the carapace (Berry & Shine 1980; Zuffi et al. 1999). However, the lack of pheromone-based preference of larger and greater body condition females suggests that those traits could not play an important role in reproductive mating processes of *M. leprosa*, or at least, that they are less important than other factors. In contrast, male *M. leprosa* preferred water pools with chemicals of females with a greater immune response. There are no previous studies in turtles on the choice of females with better immune system by males. However, in other animals, the choice of males with better health by females is mediated by chemical signals (Penn & Potts 1998; Rantala et al. 2002, 2003; Martín & López 2006). We suggest the existence of a similar mechanism in male mating strategies of *M. leprosa* turtles, where males might choose females with better immune responses that would have "better genes" and individual greater resistance to parasites or diseases (Hamilton & Zuk 1982), which can be inherited by their offspring (Zahavi 1975; Andersson 1994). This process could increase offspring fitness, similarly to that occurring with female mate preferences of *Drosophila serrata*, where females seem to gain genetic benefits (in terms of fitness) from their choice of males (Hine et al. 2002). Anyway, the results obtained in our study reflected a male's preference from chemical cues of females with better health, but no for females with larger size or with a greater body condition. This is probably explained

because body condition per se does not predict immunocompetence and health state in turtles (Polo-Cavia et al. 2010b). These data suggest that males might prefer to select on quality but not quantity of future offspring.

Trials of male scents exposed to other males showed that male *M. leprosa* avoided water with chemicals of conspecific larger males, but oriented toward water with chemicals of smaller male. In addition, this pattern was the same when considering the relative size of the pair focal-donor male. These results are consistent with those found in *E. orbicularis* where males avoid the odours of larger males and prefer to occupy water with chemical stimuli from relatively smaller males (Poschadel et al. 2006). This suggests that relatively smaller males attempt to avoid the risk of intrasexual aggression, by avoiding to occupy water pools used by larger males. In contrast, large males prefer to occupy water pools used by small males, as larger males would be victorious in agonistic encounters and could preclude mating of these small males with females in the area, thus eliminating potential competitors. In lizards, male chemicals play an important role in aggressive encounters (Alberts et al. 1994; Cooper & Vitt 1987) and can help to reduce the level of aggression (Aragón et al. 2000; López & Martín 2002, 2011). Male *M. leprosa* turtles did not show any chemical stimulus-based preference for male's health state and body condition. In some lizards, certain chemical compounds may give information on the status of dominance of a male, which may also be related to the male's immune system (Martín et al. 2007b). In our study, male turtles did not seem able to discriminate, or were not interested in, the immune system or condition of other males. This suggests that body size could be the most important factor in competitive interactions between males, while condition and immune response could not. In agreement with these results, several field studies have shown that larger turtles displaced smaller individuals more often than the converse

during basking activity (Bury & Wolfheim 1973; Bury et al. 1979; Lovich 1988; Lindeman 1999). In this way, size should be the main factor that determines dominance status in turtles (Froese & Burghardt 1974). Thus, chemical detection and avoidance of larger and stronger males may prevent agonistic encounters with these males, where smaller males could have previous disadvantage, which may allow being injured and saving energy expenditure.

In summary, we provide evidence of intra- and intersexual communication via chemical signals in water released by conspecifics in the Spanish terrapin (*M. leprosa*). Chemicals of males may convey information about their size to females and other males. Probably, male *M. leprosa* of different sizes may differ in the chemical composition of their gland secretions, or in the amount of chemicals secreted, such as it occurs in female gather snakes (*Thamnophis sirtalis*) (LeMaster & Mason 2002), or in some lizards (Alberts et al. 1994; Martín & López 2007) where skin or gland lipids composition vary with body size. On the other hand, females probably have a health condition-dependent chemical signal informing about their immunological response, as it also occurs in many other animals (Penn & Potts 1998). This would suggest that male turtles may base their mating preferences on chemical cues that honestly report the female's health quality. Further studies are needed to understand the role of chemical communication in sexual selection processes of aquatic turtles.

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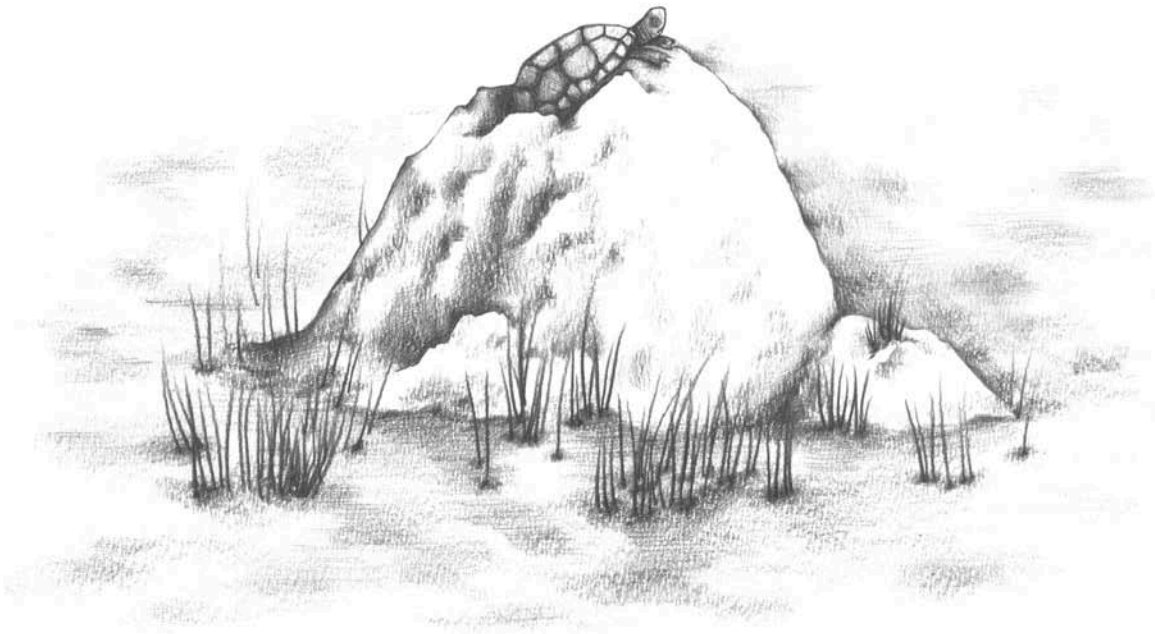
Chapter II



This chapter reproduces entirely the manuscript:

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Boldness and body size of male Spanish terrapins affect their responses to chemical cues of familiar and unfamiliar males

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Abstract: Recognition and avoidance of conspecifics based on chemical cues could reduce the risk of aggressive interactions between males. Success in agonistics encounters with unfamiliar males should be lower than with previously known familiar males. Then, males should avoid the chemicals from unfamiliar males with respect to those from familiar males. However, boldness and size could affect the outcome of encounters between males and consequently the response to chemical cues of conspecific males. We compared the time spent by male turtles *Mauremys leprosa* in water pools with chemical stimuli from unfamiliar or familiar males or with their own chemical stimuli. We also performed a behavioural test to estimate boldness of turtles in an antipredatory situation. Turtles avoided the chemicals from unfamiliar males respect to familiar and their own odours but their responses depended on boldness and size of the tested turtle. Bold turtles avoided water with chemicals of unfamiliar males but not with chemicals of familiar males, whereas shy turtles avoided chemicals of both familiar and unfamiliar males. On the other hand, large males avoided the odour from unfamiliar males but small males did not avoid water with the odour from other males. Results suggest that male *M. leprosa* can detect chemicals released to water from conspecific males, and discriminate between familiar and unfamiliar males. However, responses to these chemicals depended on boldness and body size of the responding turtle because these factors may affect intrasexual competition.

Keywords: Boldness, size, chemical cues, familiar recognition, intrasexual competition.

Introduction

Animals should adopt strategies to avoid or minimize the level of aggressive interactions, thereby reducing the associated costs of fighting (Maynard Smith 1982; Huntingford & Turner 1987; Marler & Moore 1988). By assessing in advance their potential opponents, and the own possibilities of winning, individuals may predict the outcome of a fight and initiate aggression or retreat (Maynard Smith 1982; Huntingford & Turner 1987). Probability of winning may depend on physical characteristics such as body size. For example, larger individual males have a greater fighting ability and are usually dominant in contests over smaller ones (e.g., López & Martín 2001). In addition, individuals may vary consistently in several personality traits (Réale et al. 2007), including boldness, which can be defined as the reaction of an individual to risky situations such as those occurring in encounters with predators or in agonistic interactions with conspecifc competitors. The personality of an individual may also determine its predisposition to engage in agonistic fights and, thus, its probabilities of winning a fight. For example, personality traits are related to the rank attained in a dominance hierarchy in fish (Sundstrom et al. 2004; Collete & Brown 2011), and boldness affects the outcome of fights in mice (Fuxjager et al. 2010).

When two individuals have already fought each other, prior experience may influence their fighting behaviour when they meet again. Usually, when social relationships between two familiar males have been already established, further fights in subsequent agonistic encounters will be of lower intensity or even replaced by ritualized displays (e.g., Glinsky & Krekorian 1985; Olsson 1994; Whiting 1999; López & Martín 2001). Reduced aggressions between neighbours, the so called ‘dear enemy’ recognition (Fisher 1954) should prevent escalated encounters between them, minimizing the

energy expended in agonistic interactions (Jaeger 1981; Glinski & Krekorian 1985; Qualls & Jaeger 1991). However, this requires that individuals were able to recognize each other. Therefore, the mechanisms that allow the recognition of familiar conspecifics (e.g. Carazo et al. 2008) might help to reduce the frequency and intensity of aggressive encounters.

Many species of reptiles present several recognition mechanisms, above all chemical systems that are widespread and are involved in many social interactions such as in sex and individual recognition, territoriality, intrasexual aggressions between males, and female mate choice (Mason & Parker 2010; Martín & López 2011). Chemical cues are used in intrasexual relationships between male lizards, where they can contribute to reduce costs of aggressive interactions (Carazo et al. 2007, 2008; Martín et al. 2007; López & Martín 2011). Responses to chemical cues of familiar and unfamiliar males should be different. For example, male Iberian rock lizards avoid scent marks from unfamiliar conspecific because the probability of success in an agonistic encounter with an unknown male is low (Aragón et al. 2001, 2003). Chemical cues are also potentially important in intraspecific communication of some turtles (Muñoz 2004; Poschadel et al. 2006; Galeotti et al. 2007; Lewis et al. 2007; Polo-Cavia et al. 2009). For example, males of some freshwater turtles can discriminate chemical cues of conspecifics in water, and use this information to avoid encounters and agonistic interactions with other males (Poschadel et al. 2006; Polo-Cavia et al. 2009; Ibáñez et al. 2012). Spanish terrapins (*Mauremys leprosa*) are able to recognize several characteristics of potential opponents, such as body size, through chemicals released by these in water (Ibáñez et al. 2012).

In this context, here we examined whether male *M. leprosa* turtles were able to recognize and distinguish familiar from unfamiliar males via chemical cues released to

water, and whether male turtles may modify their space use based on this information alone. We hypothesized that males should avoid pools containing water with chemicals from unfamiliar males respect to pools with water with chemicals from familiar males or with water with their own odour. We also investigated the effects of body size and boldness of male turtles on their response to chemical cues of familiar and unfamiliar conspecific males. Several field studies have shown that larger turtles displaced smaller individuals more often than the converse during basking activity (Bury & Wolfheim 1973; Bury et al. 1979; Lovich 1988; Lindeman 1999). Small male *M. leprosa* may prevent agonistic encounters, where they could have previous disadvantage, detecting and avoiding the chemicals released by larger and stronger males (Ibáñez et al. 2012). In this study, to avoid the effect of size differences, each male always was tested against the odour of other males of similar size (see Methods). We predicted that large unfamiliar males, but not small unfamiliar males, could represent a potential dangerous situation during competition for resources or agonistic interactions between males. Thus, we expected that large males avoided the chemical cues from large unfamiliar males but not the chemical cues from large familiar males or their own odour. In contrast, small males might not need to avoid the odours from other small unfamiliar or familiar males, because aggression levels between small males would be low.

Coevolution of personality traits could be driven by predation regime (Huntingford 1976; Dingemanse et al. 2007) but it also could be driven by competition between conspecifics, potentially by sexual selection (Colleter & Brown 2011). In lizards, males with larger heads are more dominant in agonistic interactions (López & Martín 2002) and spent less time inside the refuge after a predatory attack (López et al. 2005). Bold individuals with respect to social interactions may be bold also under threatening situations because of high testosterone levels (Huntingford 1976; Kagan et

al. 1988; Tulley & Huntingford 1988; Godin & Crossman 1994). Personality shown during antipredator responses is often related to social dominance in agonistic encounters (López et al. 2005; Colleter & Brown 2011). Thus, we expected that turtles that showed a bold or shy behaviour during antipredator test should act equally in response to chemical cues of other males. Hierarchy formation in turtles depends on the ability to obtain food and appeared stable over time (Froese & Burghardt 1974). We expected that during the familiarization process bold male turtles became dominant, or at least, more successful in competition than shy males. Thus, bold male turtles should avoid the chemicals from unfamiliar males but not from familiar males because they could have established a previous hierarchy with them and these bold turtles would be very likely dominant. In contrast, shy turtles should avoid the chemicals from any kind of male because they are likely to be dominant on shy males.

Material and methods

Animals and study sites

The Spanish terrapin (*M. leprosa*) is a thermophile medium size semi-aquatic turtle, which is found in south Western Europe and north western Africa (Busack & Ernst 1980; Da Silva 2002). It preferably inhabits ponds and streams with riparian vegetation, being less common in large rivers and reservoirs. Males are sexually mature at seven years old, with about 12 or 14 cm carapace length (Pérez et al. 1979).

During March 2011, we used funnel traps to capture adult male turtles in several small streams, ponds and tributaries of the Guadiana River located within dehesa oaklands (Alconchel and Olivenza, Badajoz province, SW Spain). We used a modified

version of the traditional underwater funnel traps, which include a mesh chimney that reaches from the body of the trap to the surface allowing the turtles to come to the surface to breath (Kuchling 2003; T & L Netmaking, Mooroolbark, Victoria, Australia). We baited traps with sardines and protected them from terrestrial predators by waiting in the proximity. We revised the traps every hour to collect turtles. All collected turtles were alive and did not show any sign of being stressed inside the traps.

Turtles were transported to "El Ventorrillo" Field Station, near Navacerrada (Madrid province) where experiments were conducted. Turtles were housed in individual outdoor plastic aquaria (60x40x30 cm) containing water and rocks that allow turtles to bask out of water. The photoperiod and temperature were the same as those of the surrounding area. Turtles were fed three times a week with minced meat, earthworms, and a commercial compound of fish "pellets". Turtles were held in captivity for more than two weeks before starting the experiments to allow acclimation to captivity conditions. All individuals were healthy and in good condition during the tests, and at the end of experiments, all of them had maintained or increased their body mass. Turtles were then returned to the exact locations of capture.

Male turtles were measured with a metal ruler (precision 0.1 mm). Carapace length (i.e., the greatest straight-line distance from the anterior end to the posterior end of the shell) ranged between 165 and 197 mm ($\overline{X} \pm 1$ SE = 176 $\pm$ 0.2 mm, N = 20). Individuals with carapace lengths below and above the median in the sample were classified as ‘small’ (< 176 mm, N = 10) and ‘large’ (> 176mm, N = 10), respectively.

Familiarization procedure

Familiarity was established by housing pairs of male turtles together in the same aquaria for two weeks before the experiments began. Each pair of 'familiar males' was composed of two turtles of similar body size from the same capture site (Olivenza or Alconchel). During the familiarization period, turtles basked and ate normally. Individuals basked on the brick, alone or stacked one on the top of the other, given the small emerged area available for basking. Thus, one turtle could displace the other during basking activity (Lindeman 1999; Cadi & Joly 2003; Polo-Cavia et al. 2010). Similarly, sometimes one turtle displaced or bit the other while competing for food items (Polo-Cavia et al. 2011). These observations indicated that males interacted and competed for the resources during this period. However, turtles did not show any signs of stress (e.g. stopping or reducing their eating or basking activity) and all maintained good condition.

We considered as 'unfamiliar males' those that had not been maintained together in the same aquaria during the two weeks prior to the experiments and that had been captured in two different study sites (Alconchel or Olivenza), which are separated by 25 km, within the same turtle population. In similar terrapin species home range size of males varied between 0.7 and 5.6 ha (Schubauer et al. 1990; Rowe & Dalgarn 2010). Thus, with this procedure, we were confident that males from the different capture sites had not had any interaction in the field before being captured. We cannot rule out the possibility of differences between capture locations in chemical composition of the pheromones released to water by male turtles, which might confound population with time spent together in the familiarization treatment. However, genetic- or phenotypic-dependent differences in chemical secretions of turtles between study sites are highly

unlikely. This is because both capture sites belong to the same turtle population, which is distributed uniformly through the study area occupying several freshwater systems interconnected by a main river and with almost identical ecological conditions. Therefore, we considered that any differences between study sites should be very small and should not affect to our experiment.

Boldness

The boldness of male turtles was estimated by simulating a predatory attack turning over them onto their own shell with the plastron upside. Thus, we reproduced a high risk situation where the turtle had to turn itself back to its normal position (i.e. righting response) before escaping of possible following attacks by the predator (Martín et al. 2005). We considered two stages in the righting response of turtles, the appearance time and the mechanical righting time (see below). The first time represents the behavioural decision of turtles on when starting (Martín et al. 2005), while the second one depends mainly on physical traits and physiological state of turtles (Steyermark & Spotila 2001; Elnitsky & Claussen 2006). During the appearance time, turtles entirely withdrawn into their shell had no visual information of the predator surroundings. Turtles must accurately assess the costs of remaining overturned vs. those of returning to natural position, and properly determine the optimal time to initiate righting, basing on this trade-off (Martín et al. 2005). Similarly, turtles and other animals adjust the time spent inside refuges or withdrawn within the shell so that the optimal time to emerge occurs when the costs of staying equal the costs of leaving (Sih 1997; Martín et al. 2005; Cooper & Frederick 2007; Polo-Cavia et al. 2008). Specifically, overturned turtles may suffer increased predation exposure, overheating or difficulties to breath, but righting

response requires a particular energy effort related with variables such as health and nutritional state or temperature, which may also vary over time. In this way, when starting to right (i.e., the duration of the appearance time) may be determined by the probability of righting success by turtles, which depends on individual intrinsic variables and external factors that affect their physiological response.

All the tests were performed at midday and the air temperature was always around 25 ºC (digital thermometer, accuracy of ±0.1 °C). Before the beginning of the tests, turtles were in their individual own home aquaria, where they could bask and attain an optimal body temperature. To standardize the trials, the same person (AI) performed all the tests and always followed the same procedure. The experimenter gently took a turtle from its home aquaria and handled it briefly while moving it to an open grassy area located 5 m from the aquaria. Thus, the time of handling was similar for all the tests. Then, the turtle was placed on the ground onto its carapace with the plastron upside. Turtles typically spent some time entirely withdrawn in the shell, after which they put the head partially out and scanned the surroundings. Then, turtles put the head, neck and legs entirely out from the shell, and started to right. From a hidden position and always at the same distance (5 m) from the tested turtle, the experimenter used binoculars to observe it and, by using a stopwatch to the nearest second, recorded the time spent hidden into the shell until the turtle emerged from the carapace and the eyes could be seen from above (appearance time) and the total time taken by the turtle to turn back (righting time). Each turtle was tested and turned down only one time. Because appearance time represents the time spent into the refuge (shell) before starting to escape (Martín et al. 2005; Polo-Cavia et al. 2008), we used this time to calculate a 'boldness index'. Shy males should spend more time hidden inside the refuge (i.e., shell) and delay the start of an active escape from the predator in comparison to bold

males, which should have shorter appearance times. Thus, we used the median of the appearance time to classify males as 'shy' (i.e., values above the median) or 'bold' (i.e., values below the median).

Choice of water pools

We analyzed the use by male *M. leprosa* of pools with water containing 1) their own chemical stimuli, 2) chemical stimuli of familiar males or 3) chemical stimuli of unfamiliar males. The experiments were conducted outdoor using two artificial pools (two plastic containers of 60 x 40 cm and 15 cm height) connected by stone ramps that allowed turtles to move easily from one pool to another and to bask out of water (Ibáñez et al. 2012). In each trial, one of the pools contained clean water and the other was filled with water containing chemical stimuli. Clean water was obtained from a nearby mountain spring stream that did not contain turtles. Water with different chemical stimuli came from the individual containers where turtles were kept separately. To obtain the water with chemical stimuli, we filled individual containers with clean water to about half of the container (18 l) and left the turtle there for three days. During this time, we did not provide food to avoid possible contamination of water with food chemicals. Thus, clean water was impregnated only with the scent of each turtle (Ibáñez et al. 2012).

Each focal male was tested in three different trials: "clean water vs own", "clean water vs familiar male" and "clean water vs unfamiliar male". To remove possible effects due to differences in size between the focal male and the male donor of the chemical stimuli, each male was tested with chemicals of other males of similar size. Differences in carapace length between the focal male and the male donor of the

chemical stimuli were very small and ranged between +10 to -13 mm in the familiar treatment and between +29 to -13 mm in the unfamiliar treatment. These differences did not significantly differ between treatments (GLMM; $F_{1,19} = 1.64$, $P = 0.21$) (see below). All experimental treatments were presented to each individual in random order and balanced. The positions of the pools were also randomized. The experiments were spaced in time at least two days, and none individual participated in one experiment on two successive days to avoid the possible stressful effects on the next test. Tests were only made on sunny days when the turtles were active, excluding very cloudy and rainy days. We monitored the temperature of pools at the beginning of the experiments (with a digital thermometer, accuracy of ±0.1 °C), the illumination intensity and the depth of the water column to ensure that these conditions were similar in both pools during all trials (DeRosa & Taylor 1980). After each test, all pools and ramps were cleaned with clean water and allowed to dry outdoor to avoid contamination with other smells.

Before starting each trial, turtles were allowed to bask for at least 2 h in their home containers. At the beginning of each test, a turtle was gently placed in the middle of the stone ramp linking the two experimental pools, to avoid any initial bias in choosing a pool or another. Turtles were usually very active, shifting between pools more frequently at the beginning of the experiment, and less frequently as time passed by. We used the instant registration procedure, monitoring each turtle from a hidden position for 2 h, and noting the location of the individual every 5 min (24 total records for each turtle in each test). In each record, if the turtle was inside one of the two pools (clean or chemical stimuli) it was designated as having chosen temporarily that particular pool, whereas if the position of the turtle was not clear (e.g., on the ramp), it was designated as having made no choice (Ibáñez et al. 2012).

We evaluated the "preferences" of each turtle for each chemical stimulus as the proportion of time (excluding time in the “no-choice” places) that each turtle spent in each pool (clean *vs.* chemical stimulus). Preliminary experiments showed no significant differences in time spent by turtles in two pools with clean water (Ibáñez et al. 2012).

Statistical analyses

To analyze differences in use of water pools with different chemical cues across treatments by the same individual subject turtle, we ran a three-way repeated measures analysis of variance (ANOVA) with the experimental treatment (‘own ’ vs. ‘familiar’ vs. ‘unfamiliar’) as a within subject factor, and size and boldness of the responding turtles as two between-subjects factor. We transformed data (square root) to normalize them before analysis (verified by Shapiro-Wilk’s tests) and we also tested the homogeneity of variances (Levene’s test). Post-hoc analyses were performed using Fisher’s Least Significance Difference tests (LSD) when ANOVAs showed significance.

Results

Boldness

Appearance times of turtles ranged between 2 and 305 s ($\overline{X} \pm 1SE = 68 \pm 21$ s). There were not significant differences in appearance time between males from the two study sites (one way ANOVA; $F_{1,18} = 0.22$, $P = 0.64$). Body size (carapace length) was not significantly related with appearance time ($r = 0.20$, $N = 20$, $F_{1,18} = 0.78$, $P = 0.39$). For

further analyses, males (N = 10) with appearance times below the median (i.e. < 23 s) were classified as 'bold', whereas males (N = 10) with appearance times above the median (i.e. > 23 s) were classified as 'shy'. Righting time ranged between 12 and 736 s ($\overline{X} \pm 1SE$=189 $\pm$ 51 s) and was positively and significantly related with appearance time (r = 0.83, N = 20, $F_{1,18}$ = 40.54, P < 0.0001). Thus, appearance and righting time could describe the same shy-bold continuum within this turtle species.

Responses to chemical stimuli

There were no significant overall differences among experimental treatments in the time spent by male turtles in the pool with chemical stimuli (Table 1; Fig. 1). There were not significant differences between shy and bold individuals, or between small and large individuals in overall time spent in water with scent of conspecifics (Table 1). However, boldness and body size affected significantly the responses to the different treatments as shown by the significant interaction terms between treatment and boldness and between treatment and size (Table 1). The interaction between size and boldness was not significant (Table 1).

Thus, with respect to boldness of turtles, shy turtles tended, although not significantly, to spend more time in the pool with their own chemical stimuli than in the familiar (Fisher's test, P = 0.08) or unfamiliar treatments (P = 0.06), which did not significantly differ (P = 0.75) (Fig. 1a). In contrast, bold turtles spent significantly more time in the pools with scent of familiar males than in pools with scent of unfamiliar males (P = 0.02) or with their own odour (P = 0.047), which did not significantly differ (P = 0.70) (Fig. 1a). In addition, shy turtles spent significantly less time than bold ones

in pools with chemicals of familiar males ($P = 0.03$), but they did not significantly differ in the other treatments ($P > 0.20$ in both cases) (Fig. 1a).

On the other hand, the body size of males also affected their responses to the different treatments (Table 1; Fig. 1b). Small males did not significantly differ in the time spent in the different pools with chemical stimuli among treatments (Fisher's tests: all $P > 0.27$) (Fig. 1b). In contrast, large males spent significantly less time in the pools with scent of unfamiliar males than in pools with scent of familiar males ($P = 0.027$) or their own chemicals ($P = 0.003$), which did not significantly differ ($P = 0.41$) (Fig. 1b). However, small and large males did not significantly differ in the overall time spent in the pool with chemical stimuli in the different treatments (own, $P = 0.10$; familiar, $P = 0.93$; unfamiliar, $P = 0.07$).

Table 1. Results of a repeated measures three-way ANOVA comparing the time spent by male turtles in pools with water with scent from conspecifics among the different treatments (within factor), and the effects of boldness and body size (between effects) of focal males on their responses to the different treatments. Significant effects (P < 0.05) are indicated in bold.

	F	Df	*P*
Intercept	641.71	1,16	< **0.0001**
Treatment	2.17	2,32	0.13
Boldness	0.20	1,16	0.66
Size	0.0015	1,16	0.97
Treatment* Boldness	3.39	2,32	**0.046**
Treatment*Size	3.47	2,32	**0.043**
Boldness *Size	0.28	1,16	0.60
Treatment* Boldness *Size	0.13	2,32	0.87

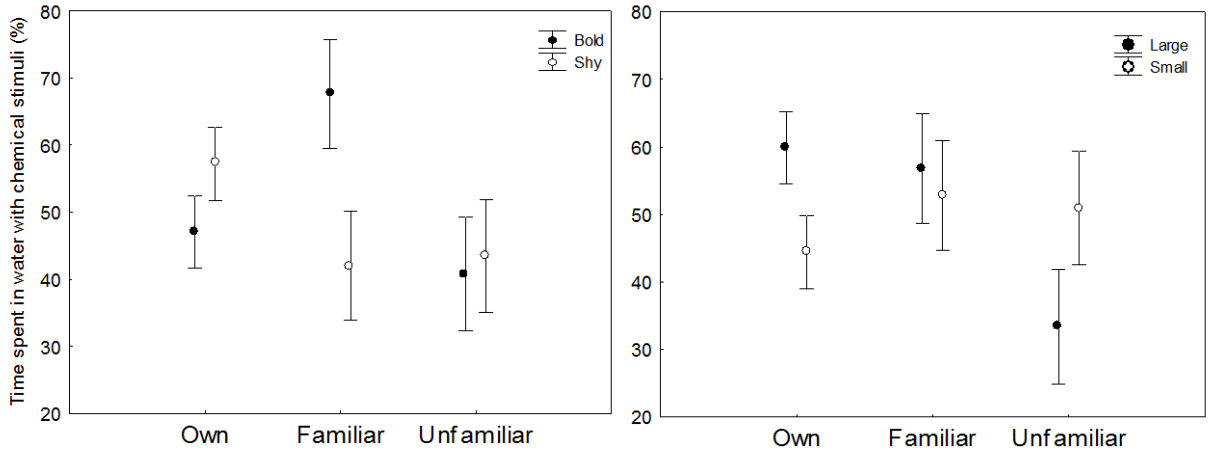

Figure 1. Percent time ($\overline{X} \pm$ SE) spent by male turtles in pools with water with conspecific chemicals in the different treatments depending on A the boldness (shy vs bold) or B the body size (small vs large) categories of the responding male.

Discussion

Our results confirm that male *M. leprosa* can detect the chemicals released to water from conspecific males. Responses to chemicals from familiar and unfamiliar males depended on boldness and body size of the responding turtle. Male *M. leprosa* classified as bold and those classified as large (both traits are independent) spent less time in water pools containing chemical cues from unfamiliar males than in the pools containing chemical cues from familiar males. These results suggest that male *M. leprosa* turtles are able to discriminate between scents of familiar and unfamiliar males. Similarly, in *Gopherus agassizii* turtles, chin gland secretions of turtles allow discrimination between familiar and unfamiliar males (Alberts et al. 1994). The ability of territorial males to discriminate between neighbours (familiar) and non-neighbours (unfamiliar) might help to stabilize social systems by reducing the frequency and intensity of aggressive encounters between males (Glinski & Krekorian 1985). Thus, the results obtained in this study suggest that recognition of familiar and unfamiliar males by male turtles *M. leprosa* could prevent escalated agonistic interactions between males.

However, responses to conspecific chemical cues were not similar for all males. Boldness affected responses to chemical cues of familiar and unfamiliar male turtles. Shy turtles spent more time in the pool with their own chemical, avoiding pools with chemicals from either familiar or unfamiliar males. In contrast, bold males preferred the chemicals of familiar males to the chemicals of unfamiliar males. In any case, shy males spent less time than bold ones in the pools with chemicals from familiar males. Boldness is associated with dominance in fishes and other animals (Sundstrom et al. 2004; López et al. 2005; Colleter & Brown 2011). In the white-footed mice (*Peromyscus leucopus*) shy or bold personality types modulate winning ability in a

context manner, suggesting that this species uses a conditional strategy to appropriately gate displays of social aggressions (Fuxjager et al. 2010). The results found in our experiment showed that male turtle's boldness in antipredatory contexts could also be related with boldness in male-male interactions. Thus, shy-bold behaviour could influence the selection of pools with chemicals from other males. Hierarchy formation during familiarity process could explain avoidance of the familiar chemicals by the shy males that could had been in a previous disadvantageous situation in agonistic encounters and competition for the resources. Otherwise, bold males preferred to stay in pools with odours of familiar males, probably because they could be victorious in agonistic interactions. In some spiders, previous experience during the male interactions could influence the outcome of the future fights and could learn to be winners or losers (Whitehouse 1997). In turtles, competition for resources such as food (Polo-Cavia et al. 2011) or basking sites (Cadi & Joly 2003; Polo-Cavia et al. 2010) could explain hierarchy formation during the familiarization process in our study. Also, familiar males could have established previously in the field dominance relationships between them because they were captured in the same or very nearby locations. Thus, both in the field and during the familiarization procedure, turtles could have experienced several fights and/or aggressions. In this context bold turtles could be more successful during agonistic interactions or competition for food or basking sites than the shy males. Both, shy and bold males had the same avoidance responses of the odors of unfamiliar males, suggesting that chemicals from unknown individual males could represent a potential dangerous situation for all males. An alternative explanation could be that, because the unfamiliar male came from a different capture site than the tested male, there might be interpopulational differences in chemical composition of sex pheromones. Thus, bold males could prefer to occupy the pools with water with chemicals of males of the same

population (i.e., familiar) than the pools with water of chemicals of males of other populations (i.e., unfamiliar) because a new different smell could represent a risky situation independently of the familiarization procedure followed in this experiment.

Size and relative size in male *M. leprosa* seem to be the most important factors, or at least more important than other traits such as body condition and health state, in modulating responses to chemicals of conspecific males (Ibáñez et al. 2012). Previous studies showed that smaller males avoid using water pools with chemicals of large males, which in contrast prefer to use water pools with chemicals of smaller males (Ibáñez et al. 2012). Similar results were observed in the turtle *Emys orbicularis* (Poschadel et al. 2006). The results of the current experiment suggest that only large males seem to recognize and avoid the water pools with chemicals from unfamiliar males. The apparent lack of discrimination between familiar and unfamiliar chemical cues by small males could be explained because in our study the small males always were tested against the odour of other males of similar small size, and large males were always tested with odours of males of similar large size. In agreement with these results, several field studies have shown that larger turtles displaced smaller individuals more often than the converse during basking activity (Bury & Wolfheim 1973; Bury et al. 1979; Lovich 1988; Lindeman 1999). Thus, the pheromones released in water from large unfamiliar males, but not necessarily from small unfamiliar males, could inform other males about a threatening situation. This difference would lead to size-dependent responses to conspecific cues (Aragón et al. 2000). These results suggest that familiarity with neighbors could allow inferior competitors to reduce the frequency and intensity of interactions (Aragón et al. 2001, 2007; López & Martín 2011).

Our results provide evidence that boldness and size could influence intrasexual competition between males. Recently, it has been shown that variation in personality

may affect female mate preferences (David & Cézilly 2011). In the same way, male-male interactions in the Spanish terrapin could depend on the individual personality along the shy-bold axis and consequently affect their responses to conspecific chemicals. Nevertheless, this is the first study to demonstrate both chemical discrimination between familiar and unfamiliar males in a freshwater turtle as well as the importance of some traits such as size and boldness in the responses to conspecific chemical cues. Future studies should focus on the influence of boldness on sexual selection, and on the role of chemical cues in modulating these processes.

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Ethical Standards Captures and experiments complied with all current laws of Spain and the Environmental Agency ("Consejería de Industria, Energía y Medio Ambiente") of the "Junta de Extremadura" Local Government (permit number: CN0008/11/ACA).

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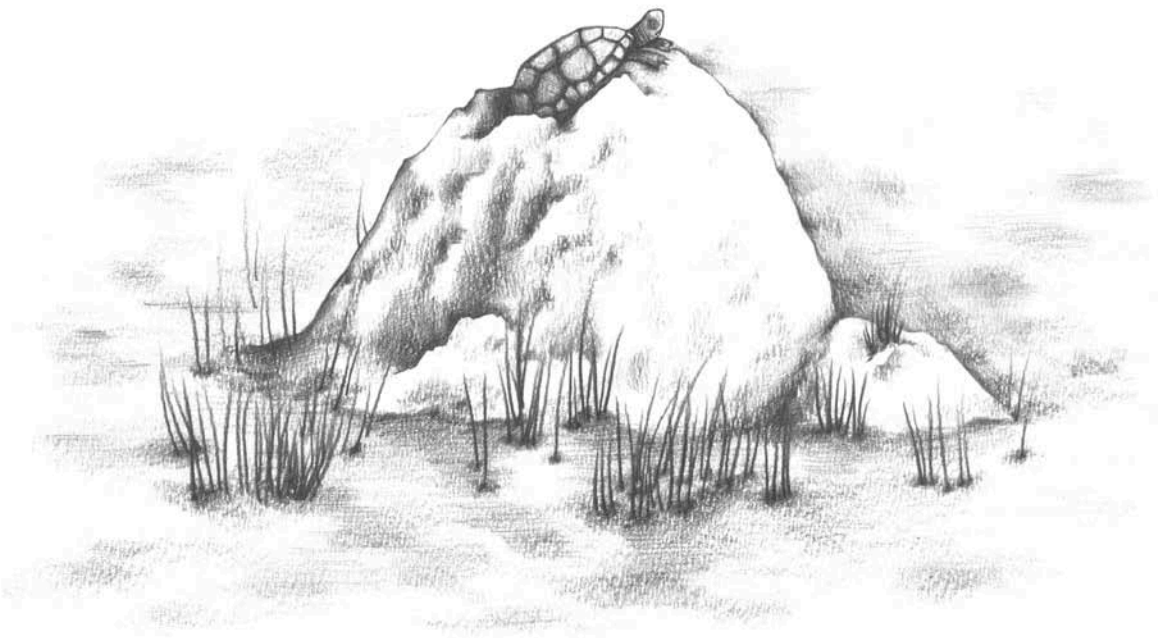
Chapter III



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Sexually dichromatic coloration reflects size and immunocompetence in female Spanish terrapins, *Mauremys leprosa*

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Abstract: Many studies have shown the importance of colorful ornamentation in mate choosiness or intrasexual conflict. However, research on color ornaments has focused mainly on birds, lizards or fish, but remains practically unknown in other animal groups such as turtles. In addition, female ornaments and their relation with sexual selection also remain almost unknown. Here, we measured the coloration of the shell and the limb stripes of male and female Spanish terrapins *Mauremys leprosa*, and explored the existence of sexual dichromatism and the relation of color characteristics with body size and health state estimated from the immune response to the injection of an antigen (PHA test). Our results showed that shell coloration, which could be constrained by natural selection to be cryptic, changed with body size, but. did not differ between sexes. In contrast, females had brighter and less UV saturated and more orange saturated limb stripes than males. In females, interindividual variation in limb stripe coloration was related with body size and immune response suggesting that this coloration may inform honestly about multiple traits that could be important in sexual selection. In contrast, coloration of limb stripes of males was duller than in females, and was not related with any trait suggesting that coloration is not important in sexual selection for males.

Keywords: Visual signals, Female ornaments, Sexual dichromatism, Immune response, Sexual selection, Turtles.

Introduction

Sexual dichomatism evolves when colorful traits are subjected to sex-dependent selection (Darwin 1871). However, coloration in secondary sexual traits is not only driven by sexual selection but also by natural selection. Thus, sexual selection may favor the evolution of conspicuous coloration, which plays important roles in mate choice, intrasexual competition, or both, while natural selection may act in the opposite way, selecting for cryptic and less showy ornaments (Endler 1980, 1983; Andersson 1994). For example, females often based their mate choice on more colorful and brighter available males in several species of fishes (Houde 1987; Kodric-Brown & Brown 1985) and birds (Hill 1990, 1991; McGraw & Ardia 2003). However, traits favored by sexual selection may sometimes be disadvantageous, or opposed by other components of natural selection (Darwin 1871; Wade and Arnold 1980). For example, guppies under high predation rates become less conspicuous, with a reduction of brighter colors, than guppies under low predation rates (Endler 1980).

Some theorical models propose that multiple different signals can give information about different male traits, so that the signals can be evaluated together to assess overall mate quality or alternatively, different receivers may pay attention to different signals and to different aspects of male quality (Reviewed in Candolin 2003). For example, in male ostriches different traits may inform about different components of the immune system, suggesting that multiple signals in sexual selection reflects different aspects of individual quality (Bonato et al. 2009). In the same way, inter-individual variation in male lizard coloration can be related to some traits such as body size, body condition, immune response, dominance and pairing status, revealing different messages to different conspecifics (Martín & López 2009). The hypothesis of

Hamilton & Zuk (1982) predicts that male birds with the most elaborate secondary sexual traits signal their higher resistance to parasites. The high energetic cost of producing and maintaining the immune system may be the link between sexual advertisement and immune system condition (Wedekind 1992; Wedekind & Folstad 1994). Many pigments found in animals, such as carotenoids, pteridines, melanins and porphyrines have antioxidant and immunoregulatory properties, and the allocation of pigments to visual signals may indicate that the signaller have enough pigments to cover both coloration and the physiological functions of these pigments (reviewed in McGraw 2005; Svensson & Wong 2011). Thus, many types of pigment-based colour ornaments in animals could honestly reveal health state via the immunomodulatory action of the pigments themselves (McGraw 2005). Individuals choose mates based on their genetic disease resistance by assessment of traits (i.e. bright colors) whose full expression is dependent of vigor and health state (Hamilton & Zuk 1982). For example, only brightly colored male birds can have an advantage in obtaining mates due to a greater immune system (Dufva & Allander 1995; Figuerola et al. 1999). However, the benefit obtained by females in such choice is not clear (Johnstone 1995). One hypothesis could be that females could benefit from choosing males with high parasite resistance if this trait is heritable. For example brighter three–spined stickleback (*Gasterosteus aculeatus*) males have offspring with greater disease resistance (Barber et al 2001).

Most studies on sexual visual signals have focused on birds, where ornamental colors that are made up of different pigments may reflect different traits; carotenoid pigments are often related with nutritional status, immune system and parasite resistance (Hill 1990; Hill & Montgomerie 1994; McGraw & Hill 2000 Mougeot et al. 2009), whereas melanin-based colors mostly reflect dominance or social status in males (McGraw & Hill 2000). Nevertheless, a meta-analysis of both types of coloration found

a lack of evidence for any significant difference between carotenoids and melanins in sexual ornamentation in birds (Griffith et al. 2006). Research on color ornaments has mainly focused on males, whereas over time the studies on the evolution of multiple signals and sexual ornamentation in females have become increasingly important (Amundsen 2000a,b; Weiss 2006; reviewed in Clutton-Brock 2007). Non-functional female ornamentation, which seems not to have direct adaptative benefits, might be a result of genetic positive correlation with male traits that are selected for intra-sexual competition or mate choice (Darwin 1871; Lande 1980; Muma & Weatherhead 1989; Cuervo et al. 1996). Thus, in most taxa, the relation between female ornaments and the sexual selection process remains almost unknown. However, it has been shown that female ornamentation could evolve through natural and sexual selection acting directly on females in some species of birds and fishes (Amundsen 2000a; Clutton-Brock 2007). For example, colorful belly of female two-spotted gobies has evolved, at least partly, as a response to male mate choice, supporting the hypothesis that female ornamentation is sexually selected (Amundsen & Forsgren 2001). Also in some lizards, different components of color ornamentation such as area and chroma may reflect different aspects of the phenotypic quality of the female and her offspring, suggesting a condition-dependent signal to potential mates (Weiss 2006; Weiss et al. 2011).

In reptiles, the role of sexual coloration has been well studied in some lizards (reviewed in Cooper & Greenberg 1992) but, although sexual dichromatic coloration is known in some turtles (Moll et al. 1981; Rowe et al. 2013; Bulté et al. 2013), its possible signalling function remains practically unknown. Only a few recent studies have shown that colorful skin patches and stripes of the head might be linked with individual quality, suggesting that coloration could evolve under sexual selection pressures in *Testudo hermanni* tortoises (Galleotti et al. 2011) and in *Trachemys scripta*

terrapins (Polo-Cavia et al. 2013). Also, darker shell coloration has been related with dominance in male tortoises, with darker eumelanic males being more aggressive during antagonistic encounters (Mafli et al. 2011). Similarly, many other turtle species have conspicuous colorations (Ernst & Barbour 1989). Nevertheless, the stripes of the head and limbs of freshwater turtles could have an alternative cryptic function rather than sexual signaling, and help to break up their outlines against vegetation when viewed by a predator from above or below.

The first aim in our study was to examine the existence of sexual dichromatism in different traits (shell and limb stripes) of Spanish terrapins *Mauremys leprosa*. Both males and females have orange-yellow stripes at head, neck and limbs (Andreu & López-Jurado 1998), which could act as honest signals in intrasexual competition and mate choice. We expected that these colorful stripes should show intersexual differences if coloration is under different sexual selection pressures in males and females. However, shell color, which is typically brown-green, should not show variation between sexes because it is exposed directly to predators, and should evolve to be equally cryptic in both sexes. We also measured body size and health- related physiological parameters (i.e., haematocrit and immune response) that could be related with dominance or quality of potential mates. We examined the relationship between these traits and limb and shell coloration. Body size and health state (i.e., immune response), signalled via chemical cues, and might be important in sexual selection of aquatic terrapins (Poschadel et al. 2006; Ibáñez et al. 2012, 2013). Individuals can use multiple signals addressed to different receivers (e.g., males or females) that can convey information about different messages (Pryke et al. 2001; Andersson et al. 2002). Thus, we expected that female and male turtles could also visually signal for different

messages to conspecifics though the characteristics of colorful ornaments which could be related to body size and health state of the signaler.

Material and methods

Study population

As a part of a long-term mark-recapture study, we captured a total of 91 turtles (65 males and 26 females), in several ponds, small streams and tributaries of the Guadiana River. Freshwater habitats of the study place are located in dehesa-oak woodland close to Olivenza and Alconchel (Badajoz province, southwestern Spain) that contain a large population of turtles. We captured turtles during March coinciding with the reproductive season of this species (Andreu and López-Jurado 1998; Arnold & Ovenden 2002). We used a modified version of the traditional underwater funnel traps, which include a mesh chimney that reaches from the body of the trap to the surface allowing the turtles to come to the surface to breath (Kuchling, 2003; T & L Netmaking, Mooroolbark, Victoria, Australia). We baited traps with sardines and protected them from terrestrial predators by waiting in the proximity. We revised the traps every hour to collect turtles. All collected turtles were alive and did not show any sign of being stressed inside the traps.

Turtles were held in captivity in a country house building close to the capture sites while we took morphological, coloration and physiological measurements (see below) and to be used in other experiments. Turtles were housed in individual outdoor plastic aquaria (60 x 40 cm and 30 cm height) containing water and rocks that allowed turtles to bask out of water. Turtles were fed minced meat, earthworms, and a

commercial compound of fish "pellets" three times a week. Photoperiod and temperature were the same as those of the surrounding area. Once all measurements were collected, turtles were returned to the exact locations of capture in good health.

Size, haematocrit and immune response

Turtles' carapace length and body mass were measured the same day of capture with a metal ruler and a Pesola spring balance respectively. Females' carapace length ranged between 111 and 224 mm ($\overline{X} \pm 1SE = 186 \pm 5$ mm, $N = 26$) and their body mass ranged between 200 and 1,550 g (947 ± 63 g, $N = 26$). Carapace length of males ranged between 127 and 197 mm ($\overline{X} \pm 1SE = 167 \pm 2$ mm, $N = 65$) and their body mass ranged between 245 and 810 g (558 ± 15 g, $N = 65$).

Blood samples were collected from the caudal sinus at the base of the tail by using a 1 mL syringe, with a 30 G, 1/2, 0.3 x 13 mm heparinized needle for each turtle (Polo-Cavia et al. 2010). Blood was placed in a heparinized capillary tube and centrifuged during 10 min at 10,000 rpm. The haematocrit (i.e., the volume percentage of red blood cells in blood) was measured with a graphical scale in each capillary tube as the ratio between the length of the capillary tube occupied by packed red blood cells and the total length of the capillary tube occupied by the blood sample. The haematocrit was not significantly related with body mass ($r = -0.10$, $N = 91$, $F_{1,89} = 1.00$, $P = 0.31$). The haematocrit is a physiological parameter that might be related with health state of turtles because it has been shown that malnourished turtles significantly decrease their haematocrit values (Tavares-Dias et al. 2009).

We measured the cell.mediated immune (CMI) response of 27 turtles (13 females and 14 males) by means of the phytohaemagglutinin test (PHA) (Smits et al.

1999; Belliure et al. 2004). This is a test 'in vivo' that estimates the delayed hypersensitivity immune response mediated by T lymphocytes, but that may also involve components of the innate and adaptive immune system (Martin et al. 2006). However, we used this index because it is a standard measure of immunocompetence and health state, regardless of the type of cells involved in it (Polo-Cavia et al. 2010). To avoid any effect of the PHA injection on haematocrit values, we performed this test some days after taking blood samples. We used a spessimeter (accuracy of 0.01 mm Digimatic indicator ID-C Mitutoyo Absolute cod. 547-301, Japan) to measure the breadth at the same marked point of the left hind limb of turtles before and 24 h after injection of 0.04 mg of PHA dissolved in 0.02 ml of phosphatebuffered saline (PBS). The general health of turtles was not affected by this test. The only noticeable effect of the injection of PHA was a slight swelling of the skin that disappeared after 48 h. We calculated the CMI response as the difference between the thickness of the leg at the injection site before and after antigen injection (Smits et al. 1999; Martín & López 2006; Polo-Cavia et al. 2010). Larger turtles tended to have increased swelling response due to the injection effect rather than a PHA specific response (Schwanz et al., 2011). In our study the CMI response was significantly and positively related with body mass (r = 0.45, N = 27, $F_{1,25}$ = 6.38, P = 0.02). Thus, we calculated the residuals of the regression between immune response and body mass in order to test the effects of the CMI response independently of size of the turtles.

Color measurements

Reflectance spectra between 300 and 700 nm were recorded using an Ocean Optics UV-VIS (JAZ-EL200) spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA) on two

traits (limb stripes and shell) of each turtle. We recorded this range of reflectances, including UV range (300-400 nm), because freshwater turtles have UV receptors that allow a tetra-chromatic color vision (Arnold & Neumeyer 1987). Mean reflectance was summarized over 5 nm steps ("binned", Grill & Rush 2000). To the naked eye, limb and neck stripes appear uniformly yellow-orange in limb and brown-green in the shell. Turtles have retractable necks and usually hid the head inside the shell when handled. Thus, due to the difficulty of measuring coloration in the neck stripes of turtles and to prevent harming turtles, we measured coloration at five haphazardly selected places of the forelimbs orange stripes (right and left) and at five haphazardly selected places of the dorsal part of the shell (i.e., carapace) trying always to measure the same or similar body points in all individuals. Then, we calculated a mean for the limbs and other for the shell, and used these values for further analyses. Reflection was recorded using a optic fiber probe with a core diameter of 400 μm (QR400-7-UV/VIS-BX; Ocean Optics Inc.) held at a 90° angle and 5 mm from the surface. Measures of reflectance were always made by the same person (AI). A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

We mathematically summarized the spectra using principal component analysis (PCA) (see Endler 1990; Cuthill et al. 1999; Grill & Rush 2000). We performed two PCAs separately for each part of the body (limb and shell) including spectra for all turtles of both sexes. The PCA summarizes all of information about the shape of complex reflectance spectra into a few PCs that are independent of one another (Montgomerie 2006). Typically, in PCA of spectral data, PC1 represents variation in brightness, and the two subsequent PCs represent chromaticity (Endler 1990; Cuthill et al. 1999; Grill & Rush 2000). Also, the PCA identifies those sections of the spectrum

(wavelength regions) that are contributing to the observed variation, independently of their "importance" in terms of contribution to the total amount of reflectance (Montgomerie 2006).

In addition to the PCA, we calculated from the raw spectral reflectance data of each body part the UV chroma (R_{UV}/R_{total}; Andersson et al. 1998), which represents relative UV reflectance. Also, to clarify the role of carotenoids in coloration, we calculated the carotenoid chroma [$(R_{700nm}-R_{450nm})/R_{700nm}$], which represents relative reflectance around peak absorbance of carotenoids, thus indicating levels of carotenoid pigments incorporated into the integument (Peters et al. 2004). We subsequently correlated UV and carotenoid chroma with the PCscores to make the interpretation of the PCAs more easily understandable,

To explore the existence of sexual dichromatism, we compared between sexes the characteristics of coloration of each trait defined by the PC scores and by UV and carotenoid chroma by using one-way analyses of variance (ANOVAs). We subsequently used backward stepwise general regression models (GRM) to examine the relationships between turtle traits (carapace length, haematocrit or immune response) (dependent variables) with characteristics of coloration of limbs or shell defined by the PC scores, including sex and the interactions between sex and the PC scores (independent variables) to examine whether there were intersexual differences in the potential relationships between turtle traits and coloration. If an interaction was significant we calculated simple regressions between trait and PC scores separately for males and females.

To assess the magnitude of the interindividual variation in the reflectance of each body part we calculated coefficients of variation (CV = s/mean) for all the reflectance across the entire spectrum (obtained from the mean of the five

measurements) of males and females separately. The CV was used to explore the function of some traits because secondary sexual traits are expected to have a greater variance than nonsexual traits (Møller 1991; Pomiankowski and Møller 1995; Cotton et al. 2004; Kelly 2005).

Results

Sexual dichromatism

The reflectance spectra of limbs or shell of males and females are shown in Fig. 1. The PCAs performed on the reflectance spectra of all individuals made separately for each trait produced three principal components each explaining more than 98 % of variation in coloration of each trait. For the limbs, pattern of coefficients relating limb-PC1 (eigenvalue = 70.1; 86.5 % of variance explained) to the original reflectance data were all negative and of similar magnitude and relatively flat across the spectra of all individuals (Fig. 2a), so limb-PC1 represented achromatic brightness variation in original spectra and the main variation source in limb color. The limb-PC2 (eigenvalue = 6.5; 8.0 % of variance) and limb-PC3 (eigenvalue = 3.1; 3.9 % of variance) were not spectrally flat (Fig. 2a) and thus represented variation in coloration (chromaticity) of the limbs. The coefficients relating limb-PC2 to the original reflectance values were positive and with maximum values below 450 nm and were negative above 450 nm. Also, limb-PC2 scores were significantly and positively correlated with UV chroma (r = 0.86; $F_{1,89}$ = 260.57; P < 0.0001) and significantly and negatively correlated with carotenoid chroma (r = -0.35; $F_{1,89}$ = 12.74; P < 0.0006). Thus, limb-PC2 represented variation in the relative amount of short- to long-wavelength reflectance (relative

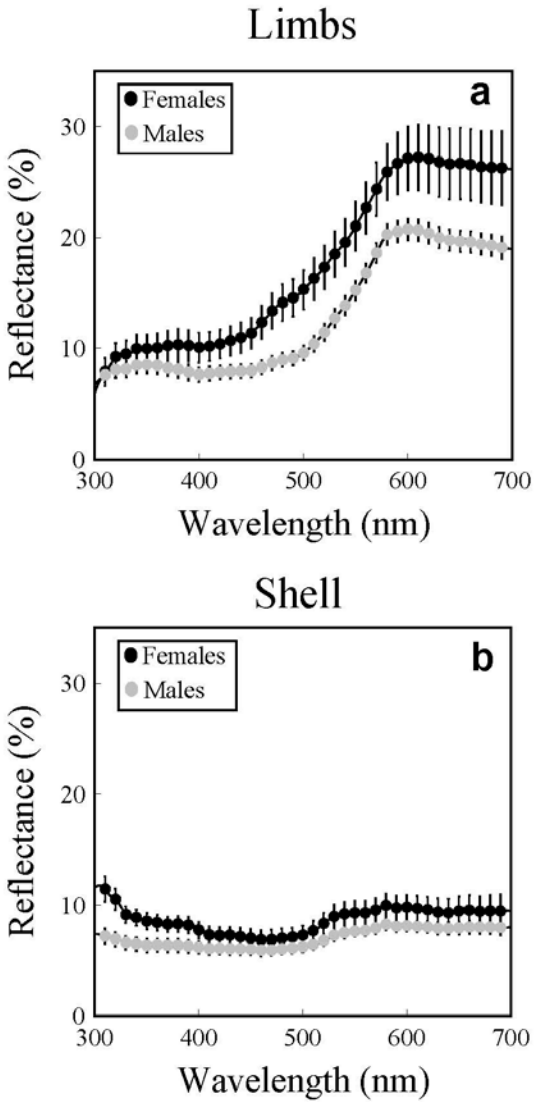

Figure 1. Mean (± SE showed at 5 nm intervals) reflectance spectra of (a) limb coloration and (b) shell coloration of female (black dots) and male (grey dots) terrapins

amount of the visual to non-visual spectra reflectance), with greater limb-PC2 scores indicating limb stripes with more UV saturated and less orange saturated, probably carotenoid-dependent, coloration. Pattern of coefficients of limb-PC3 suggested it represented variation in the relative amounts of medium (360-570 nm) wavelengths in the positive side to both short (300-360 nm) and long (570-700 nm) wavelengths in the negative side. Also, limb-PC3 scores were significantly and negatively correlated with carotenoid chroma (r = -0.71; $F_{1,89}$ = 89.12; P < 0.0001) but limb-PC3 scores were not significantly related with UV chroma (r = -0.08; $F_{1,89}$ = 0.53; P = 0.47). Thus, higher limb-PC3 scores indicated less orange saturated coloration and more greenish saturated coloration.

With respect to the shell, the first PC (shell-PC1) (eigenvalue = 67.8; 83.7 % of variance) represented achromatic brightness variation and the main variation source in shell color (Fig. 2b). Coefficients relating shell-PC2 (eigenvalue = 7.9; 9.7 % of variance) to the original reflectance values of the shell were rounding zero at long (650-700 nm) wavelengths, suggesting that shell-PC2 represented variation in relative

amounts of medium (410-650 nm) wavelengths in the positive side to short (300-410 nm) wavelengths in the negative side (Fig. 2b). Also, shell-PC2 scores were significantly and negatively correlated with UV chroma (r = -0.78; $F_{1,89}$ = 140.92; P < 0.0001) but they were not significantly related with carotenoid chroma (r = 0.19; $F_{1,89}$ = 3.38; P = 0.07). Thus, higher shell-PC2 scores indicated shells with less UV saturated coloration. Finally, the pattern of coefficients of shell-PC3 (eigenvalue = 4.2; 5.2 % of variance) were positive below 540 nm and were negative above 540 nm (Fig. 2b). Also, shell-PC3 scores were significantly and negatively correlated with UV chroma (r = -0.33; $F_{1,89}$ = 10.94; P = 0.0013) and significantly and positively correlated with carotenoid chroma (r = 0.42; $F_{1,89}$ = 18.69; P < 0.0001). Thus, shell-PC3 represented variation in relative amount of short-wavelengths (< 540 nm) in the negative side to long-wavelengths (> 540 nm) in the positive side, with higher shell-PC3 scores indicating shells with less UV saturated and more orange saturated coloration.

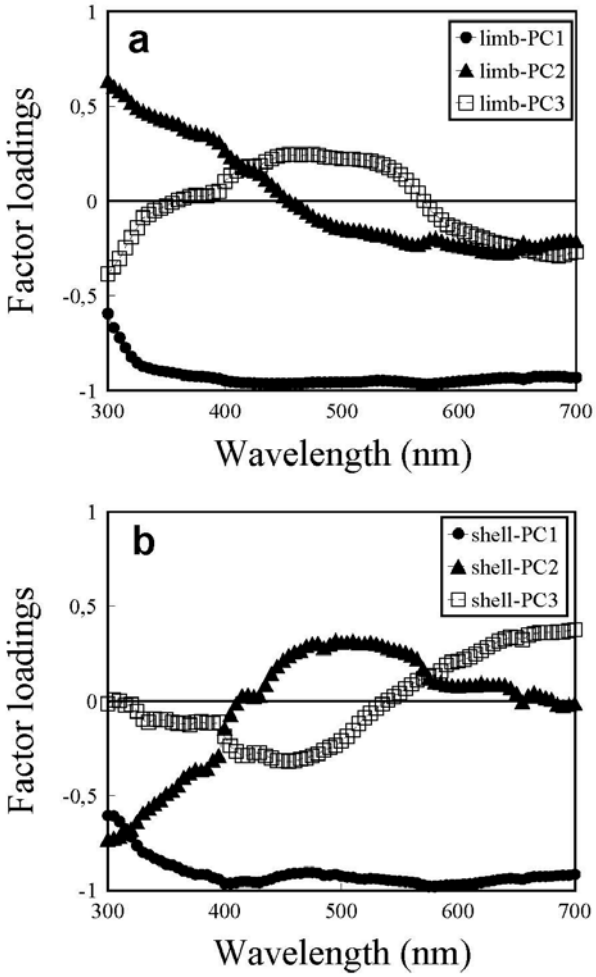

Figure 2: Coefficients of the first three principal components from PCAs on reflectance spectra that characterize (a) limb or (b) shell coloration of male and female M. leprosa *terrapins*

There were intersexual differences in limb skin coloration with males having limbs with stripes that were significantly duller (limb-PC1) and with significantly more UV saturated and less orange saturated coloration than females (limb-PC2), but there was no significant difference between sexes in limb-PC3 (Table 1). Nevertheless, coloration of limb stripes did not show sexual differences in their UV chroma and carotenoid chroma (Table 1).

In contrast, shell color characteristics defined by the shell-PCs did not significantly differ between males and females (Table 1), although males had significantly lower UV chroma in the shell than females (Table 1).

In addition, females seemed to have more interindividual variation in limb reflectance than males as showed by the relatively higher coefficients of variation of females (females' CV = 78 %; males' CV = 64 %). In contrast, coefficient of variation for shell reflectance suggested that there were no differences between sexes in the amount of interindividual variation (females' CV = 61%; males' CV = 62 %).

Table 1 PC scores (mean ± SE) resulting from PCAs on reflectance spectra, UV chroma and carotenoid chroma that characterize limb and shell coloration of male and female M. leprosa *terrapins, and results (F, P) from one-way ANOVAs comparing males and females. Significant results (P < 0.05)are marked in bold*

	Males	Females	$F_{1,89}$	*P*
Limbs:				
PC1	0.15 ± 0.12	-0.37 ± 0.19	5.24	**0.024**
PC2	0.16 ± 0.12	-0.39 ± 0.19	5.85	**0.017**
PC3	-0.08 ± 0.12	0.21 ± 0.19	1.62	0.21
UV chroma	0.14 ± 0.01	0.13 ± 0.01	0.71	0.40
Carotenoid chroma	0.58 ± 0.02	0.53 ± 0.04	1.70	0.20
Shell:				
PC1	0.11 ± 0.12	-0.27 ± 0.19	2.83	0.10
PC2	0.11 ± 0.12	-0.28 ± 0.19	2.89	0.09
PC3	0.01 ± 0.12	-0.03 ± 0.20	0.03	0.86
UV chroma	0.22 ± 0.01	0.29 ± 0.02	10.95	**0.0013**
Carotenoid chroma	0.11 ± 0.22	-0.07 ± 0.13	0.73	0.39

Relation of coloration with size and health state

Limb coloration

Body size (i.e., carapace length) differed significantly between sexes (i.e., females were larger than males) and was significantly and positively related with limb-PC2 and significantly and negatively with limb-PC3 (stepwise GRM, model: $R^2 = 0.38$; $F_{5,85} = 10.30$; $P < 0.0001$; sex effect: $\beta = 0.57$; $t = 6.23$; $P < 0.0001$ limb-PC2: $\beta = 0.35$; $t = 3.78$; $P = 0.0003$; limb-PC3: $\beta = -0.30$; $t = -3.51$; $P = 0.0007$) but the interactions between sex and limb-PCs were significant

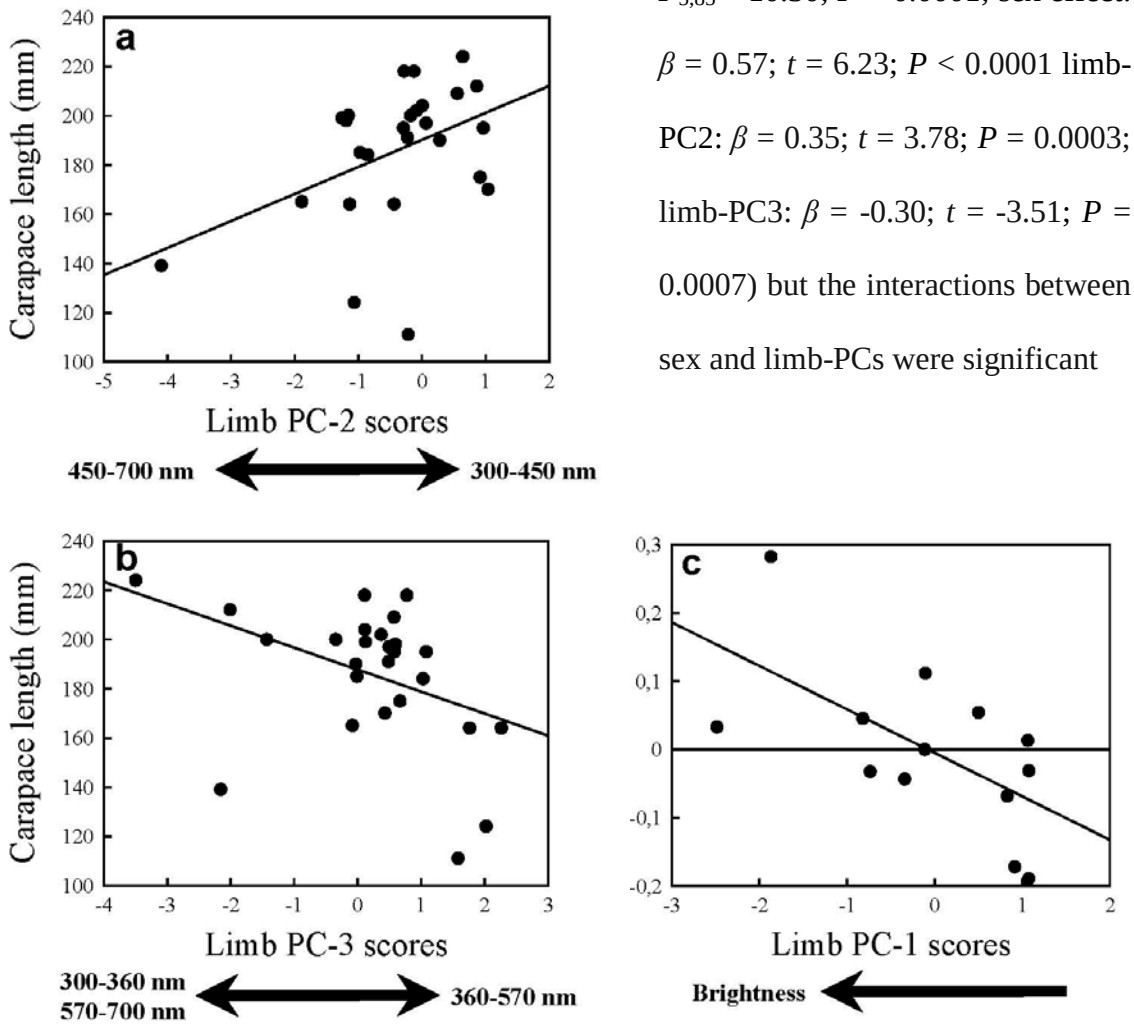

Figure 3. Relationship between (a, b) carapace length or (c) residuals of the cell-mediated immune (CMI) response on body mass and the PC scores describing characteristics of limb coloration of female M. leprosa *terrapins. Arrows indicate the variables or wavelengths correlated with each PC*

(sex*limb-PC2: β = 0.22; t = 2.46; P = 0.016; sex*limb-PC3: β = - 0.19; t = -2.23; P = 0.028). Thus, larger females (r = 0.43; $F_{1,24}$ = 5.32; P = 0.03), but not males (r = 0.16; $F_{1,63}$ = 1.77; P = 0.19), had limbs with more UV saturated and less orange saturated coloration (limb-PC2) (Fig. 3a), and smaller females (r = -0.41; $F_{1,24}$ = 4.88; P = 0.037), but not males (r = -0.17; $F_{1,63}$ = 1.86; P = 0.18), had limb coloration with more greenish saturated coloration (limb-PC3) (Fig. 3b).

The turtles' haematocrit values did not significantly differ between sexes and were not significantly related with any characteristic of limb coloration (all P > 0.05) described by the PCs, and thus no PC, nor their interactions with sex, were included in the final GRM model.

Residuals of the immune response did not significantly differ between sexes and were negatively related with the interaction between sex and limb-PC1 (stepwise GRM, model: R^2 = 0.20; $F_{1,25}$ = 6.12; P = 0.02; sex*limb-PC1: β = -0.44; t = -2.47; P = 0.02). Thus, females with higher immune responses had significantly brighter limb coloration (r = -0.58; $F_{1,11}$ = 5.68; P = 0.036) (Fig. 3c), whereas the immune response of males was not signficantly related with limb brightness (r = -0.05; $F_{1,12}$ = 0.03; P = 0.87).

Shell coloration

Body length was significantly and positively related with shell-PC2 (stepwise GRM, model: R^2 = 0.31; $F_{3,87}$ = 13.36; P < 0.0001; sex effect: β = 0.31; t = 3.45; P < 0.001; shell- PC2: β = -0.40; t = -4.33; P < 0.0001) and the interaction between sex and shell-PC1 was significant (β = -0.19; t = -2.07; P = 0.04). Thus, larger turtles, both males and females, had shells with more saturated UV coloration (shell-PC2) (Fig. 4a), and only

larger males (r = 0.40; $F_{1,63}$ = 12.20; P < 0.001), but not females (r = 0.12; $F_{1,24}$ = 0.36; P = 0.55), had shells with darker coloration (shell-PC1) (Fig. 4b).

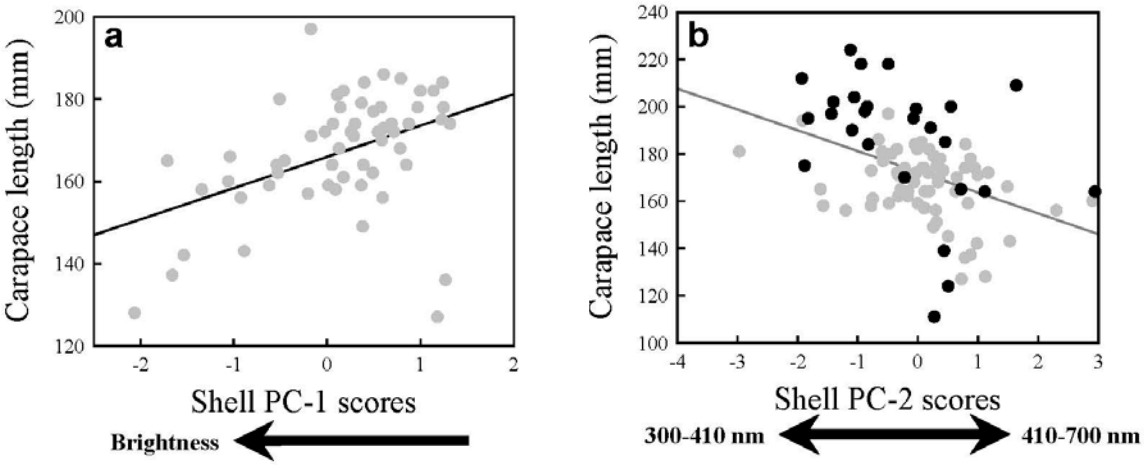

Figure 4. Relationship between carapace length and the PC scores describing characteristics of shell coloration of female (black dots) and male (grey dots) M. leprosa *terrapins. Arrows indicate the variables or wavelengths correlated with each PC*

The haematocrit and the immune response of turtles were not significantly correlated with shell coloration and thus any of the PC, nor their interactions with sex, were included in the final GRM models (all P > 0.05).

Discussion

Our results show differences in coloration between sexes of *M. leprosa* terrapins in the limb stripes (which are brighter in females), but not in the shell. Moreover, characteristics of coloration are related, differentially in males and females, with

different traits signalling body size and health state, which suggests a potential role in sexual selection.

Sexual dichromatism has been related with the degree of habitat openness in agamid lizards, suggesting that natural selection constrains the elaboration of sexual ornamentation of the body parts exposed to visual predators (Stuart-Fox & Ord 2004). Similarly, the abundance of visually oriented predator species might also provoke differences in sexual dichromatism between lizard populations (Macedonia et al. 2002, 2004). Thus, natural selection should act strongly on visible traits of turtles, such as the shell. No intersexual differences in carapace color have been found in other turtle species, which could indicate that shell coloration is involved in a cryptic function (Ross & Lovich 1992; Rowe at al. 2013). However, the head and limbs of turtles can be hidden, at least partially, by retracting them inside the shell and are not always exposed to predators. Thus, head and limb coloration could be more easily subjected to sexual selection forces than shell coloration. This could explain sexual differences in limb coloration of *M. leprosa*. Otherwise, shells have less showy and more cryptic coloration, probably because predators can detect easily exaggerate ornamentation.

Limbs of female *M. leprosa* have stripes that were brighter and with more orange and less UV saturated coloration than in males. In contrast, adult male northern map turtles (*Graptemys geographica*) express a brighter, richer, and more yellow-shifted coloration of their postorbital head spots than adult females (Bulté et al. 2013). Also, during the mating season, the head of male painted terrapins (*Callagur borneoensis*) is white with a broad, mid-saggital bright red stripe, edged in black, whereas the head of the female has a much duller uniform brown-reddish color (Moll et al., 1981). Different theories have been proposed to explain why usually in many animal species males are the brighter sex, such as less parental care of males (Trivers 1972),

higher predation on females than in males (Götmark 1992; Götmark et al. 1997) or sexual selection (reviewed in Andersson 1994). However, parental care in terrapins is at the moment unknown. We captured more males than females probably due to the greater mobility of males during early spring as they are actively searching for reproductive females. Therefore, male *M. leprosa* terrapins could be more exposed to predators during mate searching, which will favor a duller coloration to avoid being detected.

Also, coloration of limb stripes seemed to show higher interindividual variability in females than in males (i.e., higher CV for females) while shell coloration did not show differences in variability between sexes (i.e., similar values of CV). Traits subjected to sexual selection are more genetic and phenotypically variable than non-sexual traits (Pomiankowski & Møller 1995). Also, variance of sexual traits increased as condition declined (Cotton et al. 2004) suggesting that only individuals with a good condition can maintain higher degree of showiness in sexual traits. Thus, our results could suggest certain role of female limb coloration in sexual selection in *M. leprosa* (see below). Nevertheless, males of other freshwater turtles, such as *Trachemys scripta*, become drabber at larger size due to melanic changes related with size-age (Lovich et al. 1990). Thus, we cannot rule out the possibility that sexual differences in brightness of limb stripes might be due to ontogenetic melanic changes in male turtles.

On the other hand, shell coloration did not differ between sexes in *M. leprosa* terrapins. Similarly, in spotted turtles (*Clemmys guttata*) males and females do not differ in shell coloration, suggesting a possible role in crypsis (Rowe et al. 2013). In fact, shell color variation across different habitat types seems correlated with substrate ground coloration (Rowe et al. 2006a; McGaugh 2008). Nevertheless, some variations in shell coloration of *M. leprosa* might mainly reflect ontogenetic changes in size-dependent

predation risk (i.e., larger turtles have more UV saturated shell coloration). Small turtles have a higher number of bird predator species than bigger ones (Janzen et al. 2000). Of the potential bird predators of *M. leprosa* found in the study area, only storks (*Ciconia ciconia*) are able to capture adult turtles (Martín & López 1990). Then, juveniles and adults should have different pressures, compromising even more the degree of showiness of small turtles. If shell coloration evolved under natural selection pressure, we would expect smaller turtles to have less conspicuous colorations. This could explain why smaller turtles (both males and females) had a smaller amount of UV reflectance in their shells, which could render small turtles more difficult to detect by bird predators (Viitala et al. 1995; Lyytinen et al. 2001). In addition, loss of brightness in the shell of large male turtles could be related with dominance, with older and larger dominant males becoming duller. Similarly, melanic carapace coloration in males of Hermann's tortoise predicted agonistic behaviour toward other males. Thus, dark-colored carapace could be used to signal the expected degree of aggressiveness during male-male interactions (Mafli et al. 2011). However, hatchlings and juveniles of the freshwater turtles *Chrysemys picta marginata* and *T. scripta elegans* may modulate the color intensity of their carapaces making lighter or darker their shells in function of the color substrate (white or black respectively) and this process is reversible allowing the individuals to converge on the substrate color after experiencing different environments, thus facilitating crypsis (Rowe et al 2006b, 2009). Therefore, an alternative explanation could be that smaller *M. leprosa* terrapins occupy environments with lighter substrates where brighter shells could render them unperceived to predator eye.

There were also sexual differences in the traits of *M. leprosa* terrapins that were related with characteristics of coloration of the limb stripes. Males' limb coloration was not related with size or any health-dependent trait. In contrast, larger females have more

UV saturated limb stripes coloration, and females with greater immune responses have brighter limbs stripes. Thus, female ornamentation may reliably signal multiple different aspects of the phenotypic quality of the female or her potential offspring, allowing males to assess the quality of potential mates in basis of their reproductive color expression (Weiss 2006; Weiss et al. 2011). Turtles are tetrachromatic and they are sensitive to visual spectra (red, green and blue) as well as to UV wavelengths (Loew & Govardovskii 2001; Rocha et al. 2008). This opens the possibility that UV communication might be important, particularly in an aquatic environment where different wavelengths of light will be filtered out as they pass through water.

Females of other turtle species also use chemical or visual cues to signal size (Poschadel et al. 2006) or body condition (Galeotti et al. 2011; Polo-Cavia et al. 2013). On the one hand, larger females that can maintain a greater UV reflectance in their limbs could signal a greater fecundity because their larger size allows more eggs inside their body (Berry & Shine 1980). In contrast, a previous study showed that female *M. leprosa* may signal their immune quality, but not size, via chemicals released to water (Ibáñez et al. 2012). On the other hand, the physiological health state of females (i.e., the quality of the immune response) could compromise pigment allocation to sexual ornaments (Faivre et al. 2003; López et al. 2009). Similarly, a recent study with the red-eared slider (*T. scripta elegans*) showed that brightness of colorful red and yellow head patches is related with the immune response in both sexes, although the extension occupied by the red patch is larger in females (Polo-Cavia et al. 2013). Females of the lizard *Sceloporus virgatus* develop orange patches during the breeding season that might advertise egg quality and consequently offspring quality (Weiss et al. 2011). Similarly, female *M. leprosa* with brighter limbs might indicate to their mates a greater immune response that could be heritable for the future offspring. In addition to visual

cues (limb brightness), female *M. leprosa* seem also able to signal their immune response though chemical cues (Ibáñez et al. 2012), which might increase strength of preferences for possible mates (Kunzler & Bakker 2001).

In conclusion, our study showed the existence of sexual dichromatism in limb but not in shell coloration of *M. leprosa* turtles. Males were less bright than females suggesting that coloration is not important in sexual selection for males. However, limb coloration of females seems to be more variable than in males, and this variation was related with size and health state, which suggests that female coloration may be implicated in mate choice. Sexual selection forces could act strongly on female limb coloration promoting honest signals that are reliable for their receivers. Alternatively, duller coloration of limb strips in males might be linked to melanic changes related with age or size. In contrast, shell coloration did not differ between sexes, although it was related with body size in males and females. Future studies are required to clarify the function and evolution of secondary sexual ornaments in turtles.

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Ethical Standards Captures complied with all current laws of Spain and the Environmental Agency (“Consejería de Industria, Energía y Medio Ambiente”) of the "Junta de Extremadura" Local Government (permit number: CN0008/11/ACA).

Conflict of interest The authors declare that they have no conflict of interest.

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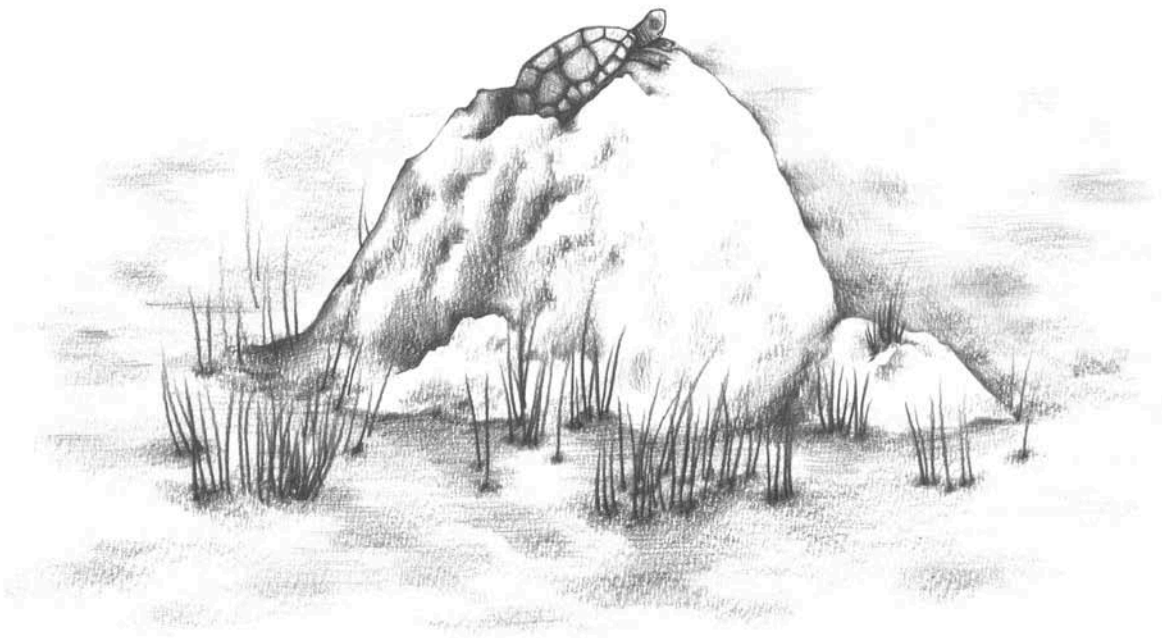
Chapter IV



This chapter reproduces entirely the manuscript:

Ibáñez, A., López, P. & Martín, J. Inter-individual variation in boldness of Spanish terrapins depends on sex, size and coloration. *Under review*.

Inter-individual variation in boldness of Spanish terrapins depends on sex, size and coloration

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Abstract: Behavioural responses to predation risk are critical for survival but since antipredator behaviour is costly, prey animals should flexibly modulate their optimum responses by considering both costs and benefits, which are partly influenced by the individual characteristics of the prey. Turtles have morphological structures that may provide partial protection, but hiding into the shell may entail very high costs. Here, we examined how gender, body size and sexual coloration influence inter-individual variability of boldness reflected in hiding behaviour of Spanish terrapins (*Mauremys leprosa*). We simulated predatory attacks of different risk level and measured the time that the turtles spent hidden entirely inside the shell (i.e. appearance times) and from then until the turtle started to flee actively (i.e. waiting times). Our results showed that when the risk increased, the appearance times were longer but the waiting times were shorter. Boldness was related with turtles' size. In addition, males appeared out of the shell earlier than females when they were previously overturned by the predator. Similarly, the conspicuousness of limb coloration was important for the appearance times of males, but not for females, and only in the "low risk" treatment. Thus, males with brighter coloration of the limb stripes demonstrated longer appearance times than duller ones, suggesting that greater visual conspicuousness increased the risk of detection. However, coloration and size had different relative importance for boldness, depending on the risk level, suggesting that when the risk level increases, antipredator behaviour should be more conservative in all individuals, rendering inter-individual differences unimportant.

Keywords: Antipredator behaviour, boldness, body size, sex, coloration.

Introduction

Predation is one of the most important selective forces that modulate the behaviour of individuals in a number of such vital activities as foraging and reproduction (reviewed in Lima & Dill 1990). Therefore, an effective antipredator strategy translates into immediate benefits for the prey by reducing mortality. Nevertheless, an exaggerated response to the predator could entail important costs in terms of time and energy allocation, with important fitness consequences (Lima 1998; Rhoades & Blumstein 2007). However, animals could reduce the costs linked to antipredator strategies with compensatory behavioural mechanisms. For example, frequent use of refuges may lead to a deteriorated body condition in lizards (Martín & López 1999a), but individuals could compensate for this cost with modifications in their hiding behaviour (Amo et al. 2007).

Aquatic turtles usually spend lengthy periods of time basking out of the water, but when they detect any approaching potential predator they dive back in, thus reducing the probability of being captured (López et al. 2005a). This escape strategy is effective in terms of avoiding the main turtle terrestrial predators, basically mammals and birds (Martín & López 1990). However, turtles may be intercepted by predators before they reach the safety of the water, this is likely, especially if they are away from it, for example when looking for nesting places, travelling between different water bodies, or during drought periods when temporary water courses dry out (Andreu & López-Jurado 1998). In this context, the shell offers partial protection (i.e. refuge) for turtles until they can escape to a safer refuge deep in the water. However, remaining hidden in the shell might carry high costs for the turtles. For example, more time spent

hiding may entail a loss of feeding and/or mating opportunities (Ydenberg & Dill 1986; Sih 1997; Martín et al. 2003a, b; Reaney 2007) or thermoregulatory costs (Martín & López 1999a, b; Polo-Cavia et al. 2009) and may also increase the risk of the predator injuring or killing the turtle, as the protection offered by the shell is only partial. Therefore, the decision as to when to emerge from the refuge (shell) and initiate an active escape may lead to a trade-off that animals should be able to optimize by modifying hiding times (Sih 1997; Martín & López 1999b; Polo et al. 2005; Cooper & Frederick 2007). Hiding times tend to increase with the costs of emerging attributable to predation risk, suggesting that the ability to assess the risk level is essential to decide when to leave the refuge (Scarratt & Godin 1992; Martín & López 1999b, 2001a, 2005; Cooper 2009). The balance between the costs of remaining hidden vs. costs of emergence should also depend on several individual traits of the prey, such as sex (e.g. Shine et al 2000; Vanhooydonck et al. 2007), body size (e.g. Krause et al. 1998; Martín & López 2003), or conspicuousness of sexual coloration (e.g. Cabido et al. 2009; Journey et al. 2013). These factors may explain the inter-individual variations in boldness under predation risks, which have often been used to define different personality types (e.g. Riechert & Hedrick 1993; López et al. 2005b; Jones & Godin 2010). In fact, natural selection, through the predation regime, may be one of the main forces modulating the evolution of personality traits (Bell & Sih 2007; Digenmanse et al. 2007).

In this study, we explored how sex, body size and sexual coloration affected inter-individual variability of boldness reflected in the antipredator hiding behaviour of Spanish terrapins (*Mauremys leprosa*) consisting in withdrawing into the shell. We simulated predatory attacks with different levels of risk and measured the time that the turtles spent withdrawn entirely into the shell, and the subsequent time after emergence

from the shell that the turtles spent immobile monitoring for predators before starting to escape actively. Because personality traits, such as boldness, may be context-dependent and could be partially explained by some biological traits (e.g. Sinn & Moltschaniwskyj 2005), we hypothesized that turtles should optimize their degree of boldness considering the risk level and also their own individual characteristics.

Firstly, *M. leprosa* individuals are able to assess predator persistence and threat degree in several antipredator simulations (Martín et al. 2005; Polo-Cavia et al. 2008). Thus, we expected that boldness depended mainly on the risk level. However, we also expected that body size may affect boldness reflected in hiding behaviour of turtles. Some models have shown the importance of the predator-prey body size ratio in their interactions (Lundval et al. 1999; Scharf et al. 2000; Aljetlawi et al. 2004). A large body could compromise the locomotor and righting response of turtles (Bonnet et al. 2001, 2010) and consequently their ability to escape actively, which in turn should affect the amount of time spent hiding in the shell after being attacked by a predator. Otherwise, an increase in size may involve other factors influencing boldness in opposite ways. For example, smaller hatchling turtles have a higher predation rate than larger ones (Tucker et al. 1999; Janzen et al. 2000). Similarly, prey vulnerability increased as size decreased in juveniles of some fish species (Scharf et al. 1998). This could force small turtles to remain hiding inside their shell for a longer time, in order to ensure that any predators in the surrounding area will have most likely gone away. However, larger turtles could be slower (e.g. lesser rate of limb movement) and more conspicuous to predator eyes than small ones. Similarly, in insects, an increase in body size may make the individual more likely to be found and/or attacked by birds (Mänd et al. 2007). Thus, we could alternatively expect that larger turtles should spend longer times hidden before they

decide to re-emerge from the shell in order to reduce the probability that the predator still remains in the surroundings and could easily detect or capture them.

Another factor that might affect predation risk is the degree of showiness expressed by individuals, because it affects the conspicuousness of prey to predators. For example, brighter and more UV-reflecting sexual ornaments may increase the probability of the individual being detected by raptors (Viitala et al. 1995; Honkavaara et al. 2002). Thus, in the absence of benefits related with showy coloration, natural selection should favour inconspicuousness against whose visual backgrounds (Endler 1984; Merilaita et al. 1999; Martín & López, 2001b). Nevertheless, sexual selection through intra-sexual competition and inter-sexual selection enhances the degree of conspicuousness in secondary sexual traits as colour ornaments favouring showy individuals (Andersson 1994). For example, female mate choice may favour colourful ornamentation in males as an honest signal (Kodric-Brown & Brown 1985; Houde 1987; Hill 1991; McGraw & Ardia 2003). Similarly, female coloration might reflect different aspects of phenotypic quality and could be implicated in male mate choice (Amundsen 2000a, b; Amundsen & Forsgren 2001; Weiss 2006; Weiss et al. 2011). Recently, some studies with chelonians showed that colourful patches and stripes on the head and limbs might act as honest visual signals that could imply some traits that are important in intra-sexual competition and/or mate choice (Galeotti et al. 2011; Polo-Cavia et al. 2013; unpubl. data). Colourful stripes on the head and limbs in turtles can be completely or partially hidden from predators' view by remaining withdrawn into the shell, whose usual cryptic coloration suggests that coloration might be modulated by both natural and sexual selection forces. Antipredatory behavioural responses should be constrained by individual conspicuousness. Thus, individuals with more striking coloration should be shyer in risky situations in order to avoid being easily detected

(Cabido et al. 2009; Journey et al. 2013). We predicted that turtles with more conspicuous colour stripes should be shyer after being attacked, and have longer hiding times, to avoid a higher probability of being detected again by the same or other predators in the surrounding area.

We also examined whether body size and coloration might affect the antipredator behaviour in males and females differently. The investigated terrapins show sexual size dimorphism, with females being larger and with brighter skin coloration than males (Muñoz & Nicolau 2006; Ibáñez et al. unpubl. data). We predicted that sexual size-related differences in locomotor performance (Bonnet et al. 2010) and different visual conspicuousness could affect the hiding behaviour of males and females in different ways. Due to this constraint, females should be shyer and need to spend a longer time hidden in the shell to be sure that the predator does not remain in the surrounding area upon re-emergence. Finally, we expected that gender, size and coloration could be of relatively different importance depending on the risk level. When the risk level increases, antipredator behaviour should be more conservative in all individuals, rendering inter-individual differences unimportant in determining boldness during hiding behaviour.

Material and methods

Animals of study and husbandry

In 2011 we captured 36 adult *M. leprosa* turtles (16 males and 20 females) in several small streams, ponds and tributaries of the Guadiana River located within the dehesa oaklands (Alconchel and Olivenza, Badajoz province, SW Spain). We made the

captures in June, at the end of the breeding season of the species to minimize the impact on their reproduction. We used a modified version of the traditional underwater funnel traps, which include a mesh chimney that extends from the body of the trap to the surface, allowing the turtles to come to the surface to breathe (T & L Netmaking, Mooroolbark, Victoria, Australia; Kuchling 2003). We baited the traps with sardines and protected them from terrestrial predators by waiting in the proximity. We checked the traps every hour to collect turtles. All the collected turtles were alive and did not show any signs of being stressed inside the traps. We used a metal ruler (1 mm precision) to measure the carapace length as the greatest straight-line distance from the anterior to the posterior end of the shell (males: $\overline{X} \pm 1SE = 175 \pm 2$ mm, range = 165-194 mm; females: $\overline{X} \pm 1SE = 190 \pm 3$ mm, range = 168-209 mm). We used a Pesola spring scale to measure their body weight (males: $\overline{X} \pm 1SE = 613 \pm 24$ g, range = 490-810 g; females: $\overline{X} \pm 1SE = 952 \pm 40$ g, range = 680-1,300 g).

The turtles were then transported in plastic cages (80 x 40 cm and 50 cm height) to "El Ventorrillo" Field Station near Navacerrada (Madrid province), where the experiments were conducted. The four-hour journey was made by car and a constant temperature of 23 ºC was maintained to avoid heat stress. We did not observe any effect of the transport on the mortality or health condition of the turtles. The turtles were housed in individual outdoor plastic aquaria (80 x 40cm and 50 cm height), containing water and rocks that allowed the turtles to bask out of the water and offered them protection in case of adverse weather conditions. The photoperiod and temperature were the same as those of the surrounding area. Three times a week the turtles were fed minced meat, earthworms, and a commercial compound of turtle "pellets". The turtles were held in captivity and the investigator minimized contact with the animals before and during all behavioural experiments to avoid possible effects of habituation. All

individuals were healthy and in good condition during the tests. At the beginning of August, when the experiments were finished, the turtles were returned to the exact locations of capture.

Colour measurements

Spanish terrapins have apparently uniform orange-red stripes on their limbs and neck, which are retractable and can be hidden within the shell in a risk situation. However, while the head and neck can be totally hidden into the shell, limbs can only be partially hidden and could still be seen by predators while turtles remain withdrawn into the shell. Thus, we measured coloration at five random places of the forelimbs orange stripes (right and left) of each turtle. Reflectance spectra between 300 and 700 nm were recorded using an Ocean Optics UV-VIS (JAZ-EL200) spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA). Reflection was recorded using a probe held perpendicular to the surface, and reflectance was always measured by the same person (AI). A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait. Mean reflectance was summarized over 5 nm steps ("binned", Grill & Rush 2000). Then, we calculated the average for the five measurements of each turtle.

We performed a PCA for limb coloration including mean spectra for all turtles and used the PC scores values for further analyses (Cuthill et al. 1999). The PCA produced three principal components. The patterns of coefficients relating limb-PC1 (eigenvalue = 57.7; 71.2 % of variance explained) to the original reflectance data were all negative and of similar magnitude and relatively flat across the spectra of all individuals, so limb-PC1 represented achromatic brightness variation in original spectra,

with turtles with lower limb-PC1 scores having brighter limb coloration. The limb-PC2 (eigenvalue = 15.7; 19.4 % of variance) and limb-PC3 (eigenvalue = 5.9; 7.3 % of variance) were not spectrally flat and thus represented variation (hue and saturation) of the limbs coloration. The coefficients relating limb-PC2 to the original reflectance values were high and positive below 490 nm and were negative above 490 nm. Thus, limb-PC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower limb-PC2 scores indicating limb stripes with more saturated orange coloration. The pattern of coefficients of limb-PC3 suggested it represented variation in the relative amounts of medium (400-570 nm) wavelengths in the positive side to both short (300-400 nm) and long (570-700 nm) wavelengths in the negative side. We used individual PC scores resulting from this PCA to characterize coloration in further analyses.

Hiding behaviour

We performed three different behavioural tests simulating predatory attacks with three different risk levels ("low", "medium" and "high"). Every turtle (N = 36) was tested in all treatments and the order of presentation was randomized. In the first treatment ("low risk"), we took one turtle from its home cage, handled it once, briefly and gently, and then released it in the prone position in the middle of an open field with short grass. For the "medium risk" treatment, we followed the same procedure as above, but turtles were released in the grass field placed on their carapace with the plastron upside. Thus, we simulated a predator that had turned over the turtle to prevent it from escaping and, therefore, to attack it more easily. In the third treatment ("high risk"), we took the turtles out of their own aquaria and walked and tapped them gently with the hand during 5 min,

simulating that a predator had captured it, before releasing the turtle placed on their carapace with the plastron upside in the grass field. To avoid other confounding effects that might affect the turtles' risk perception (Burger & Gochfeld 1993; Cooper 1997; Cooper et al. 2003), the same person wearing the same clothing performed all the tests following the same procedure. The turtles were used only once in each trial to avoid stress; the trials were spaced sufficiently (at least 1 day) so that fatigue resulting from one test did not affect subsequent tests. Before the trials, we allowed the turtles to bask in their outdoor home cages for at least 2 h, which allowed them to attain and maintain an optimal body temperature within the activity and preferred temperature range of the species (Andreu & López-Jurado 1998; Keller & Busack 2001). After the simulated attacks, the experimenter retreated and remained immobile observing with binoculars from a hidden position situated 5 m far from the turtle. After the simulated attack, and as a consequence of handling, turtles typically remained immobile and withdrawn entirely into the shell (i.e. the head, legs and tail were not or were only barely visible from above the carapace). We then measured the time that the turtle spent withdrawn into the shell, since we released it on the ground until the head emerged from the shell (i.e. when the eyes could be seen from above the shell: "appearance time"), and the time from appearance until the turtle emerged entirely from the shell and started to escape actively (i.e. when the turtle first touched the ground with the limbs or head: "waiting times"). We chose these two time measurements because "appearance time" represented the time that the turtles remained entirely hidden and had no visual information on the predator or the surrounding habitat (Martín et al. 2005). Conversely, during "waiting times" the turtles were able to monitor their surroundings visually and evaluate when to switch to an active escape (Martín et al. 2005; Polo et al. 2011).

Statistical analyses

To explore the variation in appearance and waiting times across risk level treatments by the same individual subject turtle, we used one-way repeated measures analyses of variance (ANOVAs) with risk level as a within-subject factor ("low" vs. "medium" vs. "high"). We Box-Cox transformed the appearance and waiting times to normalize them (Kolmogorov-Smirnov, $P > 0.05$) and the Levenne test ensured that variances were homogenous. Then, we performed separated General Linear Models (GLM) for appearance and waiting times within each risk level, with weight and the PC scores describing characteristics of coloration as covariates, and sex as a fixed factor. All variables were initially included in the models, and then non-significant variables were dropped until the final models contained only significant terms. When interactions between sex and other covariates occurred, we ran simple linear regressions between hiding times and those covariates separately for each sex. Post hoc analyses were performed using Tukey's tests when ANOVA or GLM showed significance. All statistical analyses were performed using the statistical package Statistica 8.0 (StatSoft Inc., Tulsa, OK. USA).

Results

Boldness variation in hiding behaviour across risk levels

The time spent by the turtles hidden entirely in their shells after a predatory attack (i.e. appearance time) differed significantly across the three different levels of risk (repeated

measures one-way ANOVA, $F_{2,70}$ = 63.11, P < 0.0001; Fig. 1). Post hoc pairwise comparisons showed that turtles had significantly shorter appearance times when they were placed in the prone position in the "low risk" treatment than when they were overturned in the "medium risk" and "high risk" treatments (Tukey's tests: both P < 0.001), and shorter appearance times in the "medium risk" than in the "high risk" treatment (P = 0.002).

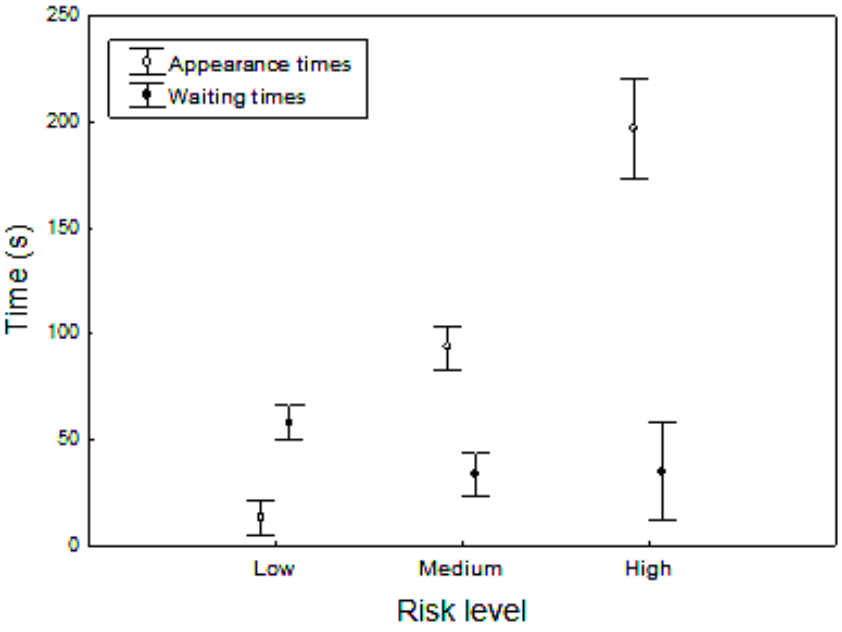


Figure 1. Mean ± SE appearance and waiting times (s) of the turtles in the "low", "medium" and "high" risk treatments

Similarly, there were significant differences in waiting times of turtles across risk levels (repeated measures one-way ANOVA, $F_{2,70}$ = 5.35, P = 0.007; Fig. 1). Post hoc pairwise comparisons showed that turtles had significantly longer waiting times in the "low risk" than in the "high risk" treatment (Tukey's test: P = 0.007). Differences between waiting times in the "medium risk" and "high risk" treatments only approached significance (P = 0.06), and waiting times in the "low risk" and "medium risk" treatments did not significantly differ (P = 0.70).

Effects of sex, coloration and size on boldness

Low risk treatment

When the risk was "low", appearance times of turtles were significantly affected by their body weight, with heavier turtles spending longer times hidden in their shells (GLM, model: R^2 = 0.19, $F_{2,33}$ = 5.10, P = 0.011; Weight effect: $F_{1,33}$ = 6.14, P = 0.018; β = 0.39, t = 2.47, P < 0.01; Fig. 2). Also the brightness of limb coloration (limb-PC1) significantly affected appearance times of turtles but depending on their gender (Sex*Limb-PC1 effect: $F_{1,33}$ = 6.41, P = 0.016).

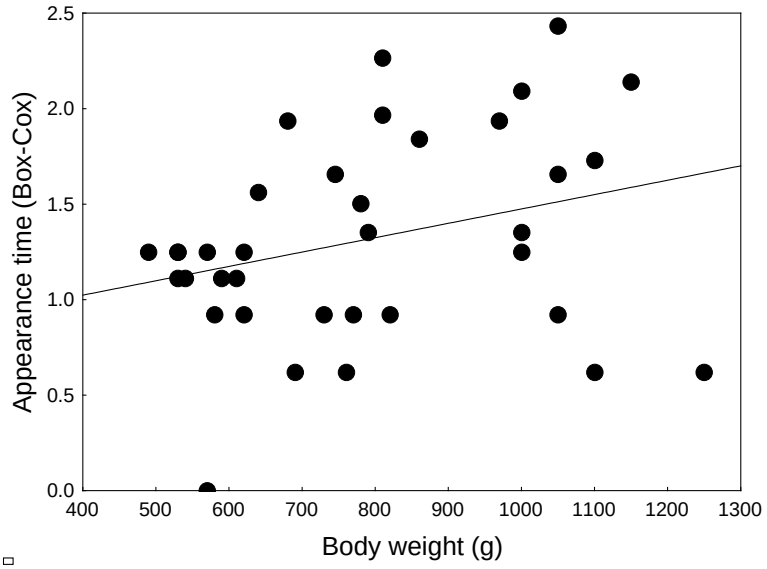
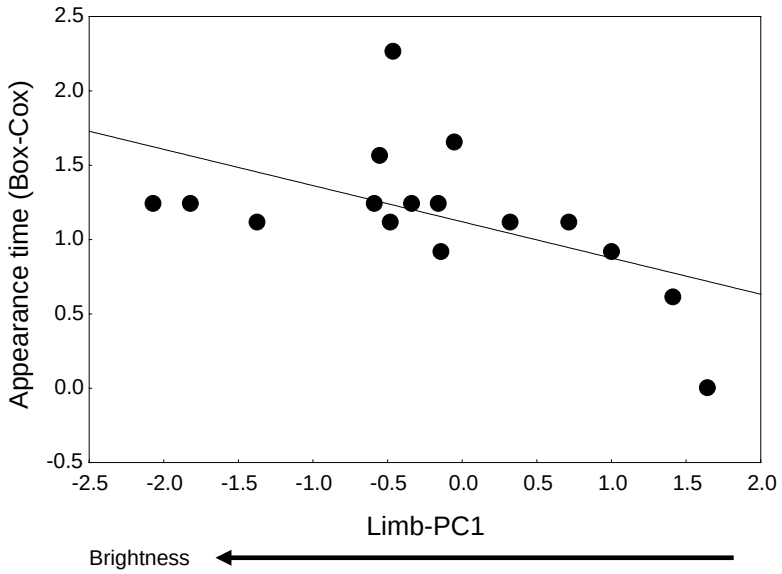


Figure 2. Relationship between appearance times (Box-Cox transformed) and body weight of the turtles after a simulated predator attack in the "low" risk treatment

Other variables had no significant effects on appearance times and were excluded from the final model (all P > 0.28). Separated analyses showed that male appearance times were significantly and negatively correlated with limb-PC1 scores (Simple regression, model: R^2 = 0.23, $F_{1,14}$ = 5.59, P = 0.03; limb- PC1: β = -0.53, t = -2.36, P = 0.03; Fig. 3). Thus, males with brighter limb coloration were shyer and had longer appearance times after a predatory attack. In contrast, limb coloration of females

did not seem important in determining the appearance times (Simple regression; R^2 = 0.02, $F_{1,14}$ = 0.55, P = 0.46).



The turtles' waiting times were not significantly affected by any of the considered variables and were not included in the final model (all P > 0.09).

Figure 3. Relationship between appearance times (Box-Cox transformed) and brightness of coloration (limb-PC1) of male turtles after a simulated predator attack in the "low" risk treatment

Medium risk treatment

Males had significantly shorter appearance times than females in the "medium" risk treatment (Males, $\overline{X}$ ± 1SE = 60 ± 19 s; Females, 121 ± 17 s; GLM, R^2 = 0.12, $F_{1,34}$=5.89, P = 0.02) but appearance times were not significantly related with size or any PC describing limb coloration (all P > 0.24; i.e. not included in the final model). Thus, in the "medium risk" treatment, females spent longer times than males hidden inside their shells until they re-emerged, independently of size and coloration.

Waiting times were significantly and negatively correlated with body weight of the turtles (GLM, R^2 = 0.12, $F_{1,34}$ = 5.65, P = 0.02; β = -0.38, t = -2.38, P = 0.02; Fig. 4). However, the effects of sex and the PCs describing limb coloration were not

significant and were removed from the final model ($P > 0.26$ for all). Thus, after re-emergence from the shell, larger turtles started to right sooner than smaller ones, independently of their sex and coloration.

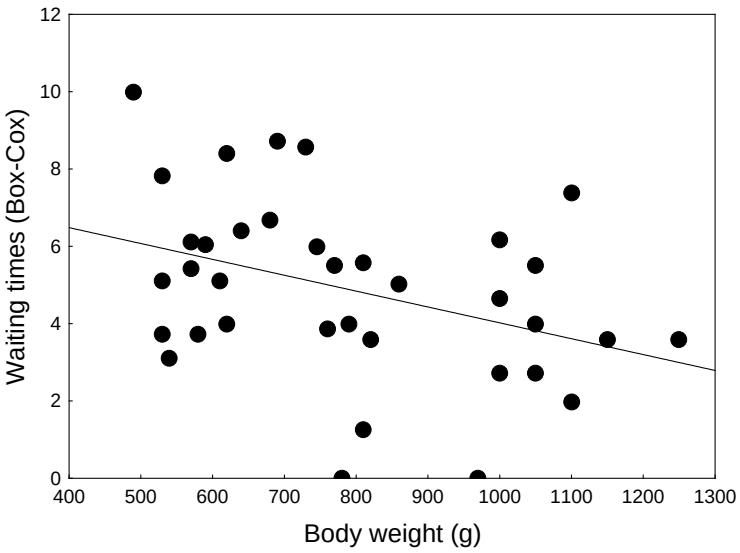


Figure 4. Relationship between waiting times (Box-Cox transformed) and body weight of the turtles after a simulated predatory attack in the "medium" risk treatment

High risk treatment

In contrast, appearance times in the "high risk" treatment did not significantly differ between sexes and were not significantly related with size or any PC describing limb coloration (GLM, all $P > 0.18$, i.e. not included in the final model).

Similarly, the waiting time spent by the turtles from the time of re-emergence from the shell to the time they started an active escape was not significantly related with any variable initially included in the model (GLM, all $P > 0.10$).

Discussion

Our study showed that *M.leprosa* turtles differed in their degree of boldness reflected in anipredatory hiding behaviour across different risk levels. Similarly to previous studies (Martín et al. 2005; Polo-Cavia et al. 2008), turtles seemed able to assess the risk level and modify their appearance or waiting times accordingly. Appearance times

differed between treatments, with shorter times spent by turtles hidden inside the shell when they were prone ("low" risk) than when they were overturned ("medium" and "high" risk). Similarly, when turtles were overturned, they had longer hiding times until their appeared from the shell when risk increased, which the turtles could infer from being handled by the predator for a longer time. In contrast, the pattern obtained for waiting times was the opposite, with longer waiting times when the turtles were prone and shorter when the risk increased. Longer appearance times from the refuge (in this case from the shell) tend to increase the costs associated to refuge use, such as loss of time and thermal costs (Martín & López 1999b). Unlike appearance times, during waiting times turtles may monitor and obtain information about the surroundings assessing whether a predator is still present (Polo et al. 2011). Thus, there are different apparent trade-offs between both appearance or waiting times and the risk level. In the "low" risk situation, turtles spent shorter times hidden inside the shell (refuge) and then longer times monitoring whether the predator is not present before deciding to escape. Thus, although a low risk attack initially only requires a low intensity antipredatory response (i.e. short appearance time), turtles might require longer times monitoring the surroundings due to the uncertainty provoked by a low-risk predator approach that could be or could not be followed by future successive attacks (Polo et al. 2011). However, while risk increases, longer appearance times and shorter waiting times might be a more effective tactic to avoid the predator because during waiting times, the turtles should just need to confirm that the predator is not waiting in the area to ambush them before they initiate escape (Polo et al. 2011). The results obtained in our study suggested that turtles shift to behavioural costly tactics (i.e. longer appearance times) under perceived high risk conditions, but that uncertainty about predation risk (in the low risk situation) may also increase costs of

refuge use due to the need to monitor the actual risk of the potential predator before deciding to switch to an active antipredatory escape strategy.

The impact of inter-individual differences in body size and limb stripes showiness on turtles' boldness varied between risk levels and affected males and females in different ways. Turtles showed a size-dependent hiding response when they were prone placed ("low" risk) with heavier individuals spending longer times inside their shells after the attack. Similarly, smaller fish also emerge earlier from their refuges than larger ones because they cannot afford the metabolic expenditure associated with hiding inside the shelter (Krause et al. 1998). Furthermore, small juveniles of Iberian rock lizards (*Iberolacerta cyreni*) have shorter emergence times from the refuge because the thermal costs of remaining inside the refuge are higher than for the relatively larger adults (Martín & López 2003). As in other freshwater turtles, *M. leprosa* males are smaller than females and this sexual size dimorphism has been related with greater agility and mobility (Berry & Shine 1980; Bonnet et al. 2001; Lindeman 2008). Thus, smaller turtles could be faster, due to greater limb movement rate, than larger ones. Nevertheless, in this study the size-dependent pattern was the same for males and females (i.e. there was no significant interaction Body size*Sex). Alternatively, larger turtles might be more conspicuous and have a greater probability of being detected by predators. Thus, greater limb movement rate or/and lower probability of being detected might allow shorter appearance times that may result in lower metabolic/thermal costs for smaller turtles.

In addition, brightness of limb stripes was related with appearance times in males, but not in females, and only when risk was low. Males with brighter coloration of limb stripes spent longer times hidden inside their shells in the "low" risk treatment. Males are less showy because they have duller orange limb stripes than females

(unpubl. data). The local abundance of visually oriented predators might drive differences in sexual dichromatism across lizard populations (Macedonia et al. 2002, 2004). Usually, body regions exposed directly to predators have less contrast with respect to the background to avoid being easily detected by predators (Stuart-Fox & Ord 2004). The stripes on turtles' limbs can be partially hidden inside the shell after a predator attack but, when the turtles decide to escape, the limbs are again exposed to predators. Males having bright colours render showiness and might result in greater susceptibility to predators (Endler 1980; Martín & López 2001b; Stuart-Fox et al. 2003). Nevertheless, individuals that are easily detected by predators due to their greater showiness may modify their behaviour and be more elusive. For example, more showy individual males of *Iberolacerta monticola* lizards behaved more cautiously, hiding for longer in refuges when they were threatened by a potential predator (Cabido et al. 2009). Similarly, bird species that have more conspicuous plumage coloration may compensate for their conspicuousness by being more responsive to the sound of predators (Journey et al. 2013). However, compared to males, the brighter colours of female *M. leprosa* turtles (Ibáñez et al. 2013) might pose a smaller predation risk to them, due to their larger size, which makes it more difficult for the locally prevailing predators to actually prey on them. Therefore, the coloration of females might be under weaker natural selection than the bright coloration of males. It could be very difficult for the potential bird predators found in the study area (Martín & López 1990) to capture an adult female turtle due to their larger size in comparison to males, suggesting that females probably have different predators than males. Similarly, the smaller size of males could render them more vulnerable to predators and showy individuals might be shyer and have longer hiding times due to their higher risk of being detected by predators (Endler & Greenwood 1988; Mand et al. 2007). The fact

that brighter coloration in females may signal a greater immune response to potential mates (unpubl. data) suggests that sexual selection might be the force that modulates limb coloration in females, while natural selection (i.e. a higher predation risk) could act strongly against the relatively smaller males having more conspicuous limb stripes.

There were sexual differences in appearance times when the turtles were overturned by the predator. Males spent less time than females inside their shells before they reappeared. Results from previous studies on the influence of sex on some components of the righting response of turtles show some discrepancies. Some studies do not find sexual differences in appearance, waiting or latency times of adult turtles when they are overturned (Martín et al. 2005; Polo-Cavia et al. 2008, 2012). On the other hand, female juveniles of sliders, *Trachemys scripta*, have longer latency times than males but they do not differ in terms of the time needed to right (Delmas et al. 2007). In addition, males of *Testudo horsfieldii* turtles are more successful and are faster in righting than females, supposedly due to their relatively long legs, higher degree of freedom to move them, a more domed dorsal part of the carapace, and bigger head (Bonnet et al. 2001). Our results with *M. leprosa* showed that males appeared earlier from the shell than females perhaps because their morphological profile might allow them better righting and escape performance. Thus, females could be less successful and slower than males in returning to their normal position, as it occurs in *T. horsfieldii* (Bonnet et al. 2001), and appear later from their shell, which in fact could give them a greater chance of a satisfactory escape if the predator still remained in the surroundings.

Otherwise, waiting times were not affected by either sex or limb coloration but heavier turtles started an active escape earlier than smaller ones in the “medium” risk treatment. Previous studies showed that *M. leprosa* had longer waiting times when the

predator is close and the response occurs in land respect to water suggesting that waiting times in this species were affected by predator persistence and the availability of safe refuges (Martín et al. 2005; Polo-Cavia et al. 2008). In our study, the observer was always situated at the same distance from the turtles used in the experiment (approx. 5 m) and all trials were performed on land. Supposedly, smaller turtles could be more vulnerable than larger ones, and this might force them to scan the surroundings accurately for longer to ensure the absence of predators before escaping. Otherwise, remaining overturned may entail high costs, such as the loss of feeding and mating opportunities and/or overheating (Ydenberg & Dill 1986; Sih et al. 1990; Martín et al. 2003; Polo-Cavia et al. 2009, 2012). Alternatively, turtles may habituate to aversive stimuli after repeated exposure (Hayes & Saiff 1967). In this way, larger turtles are also older than smaller ones and consequently more experienced to adopt a different strategy (i.e. bolder behaviour). This fact, together with the reduced ability of larger turtles to flip over after being overturned, lower predator vulnerability and the costs associated with remaining overturned might favour shorter waiting times in larger turtles.

In conclusion, appearance times increased in concordance with the risk perceived by the turtles, which behaved more cautiously when perceived predation risk increased. However, waiting times decreased when risk increased suggesting that the turtles needed more time to assess and monitor whether a predator was still present when the uncertainty of future risk was greater (Polo et al. 2011). Therefore, individuals of *M. leprosa* were able to assess the risk level in their environment and optimize their antipredator hiding response. The turtles showed a size-dependent response, with longer appearance times for larger turtles when they were prone. For males, limb brightness was the main factor affecting their appearance times, but only in the “low” risk

treatment. Nevertheless, appearance times when turtles were overturned were not affected by size but males re-emerged from their shells relatively sooner than females. Waiting times were affected by size in the "medium" risk treatment, with smaller turtles starting to right later than larger ones. Taken together, these results suggest that males and females differ in terms of the traits that influence their boldness in risky contexts in different ways and that different aspects of the antipredator behaviour might be influenced by different factors (Delmas et al. 2007). Similarly, conspicuousness to predator eyes (i.e. larger size and brighter colours) might lead to differences in appearance times in terrapins. Nevertheless, while the risk level increased, the turtles spent longer times inside their shells and needed less time to initiate an active escape and the relative contribution of size and coloration was less important (i.e. no relation in "high" risk treatment). Thus, when the risk increases, a turtle should assume that it has already been detected and then traits like body size or the degree of conspicuousness should not be important for their boldness in hiding behaviour, making the inter-individual differences in those traits irrelevant.

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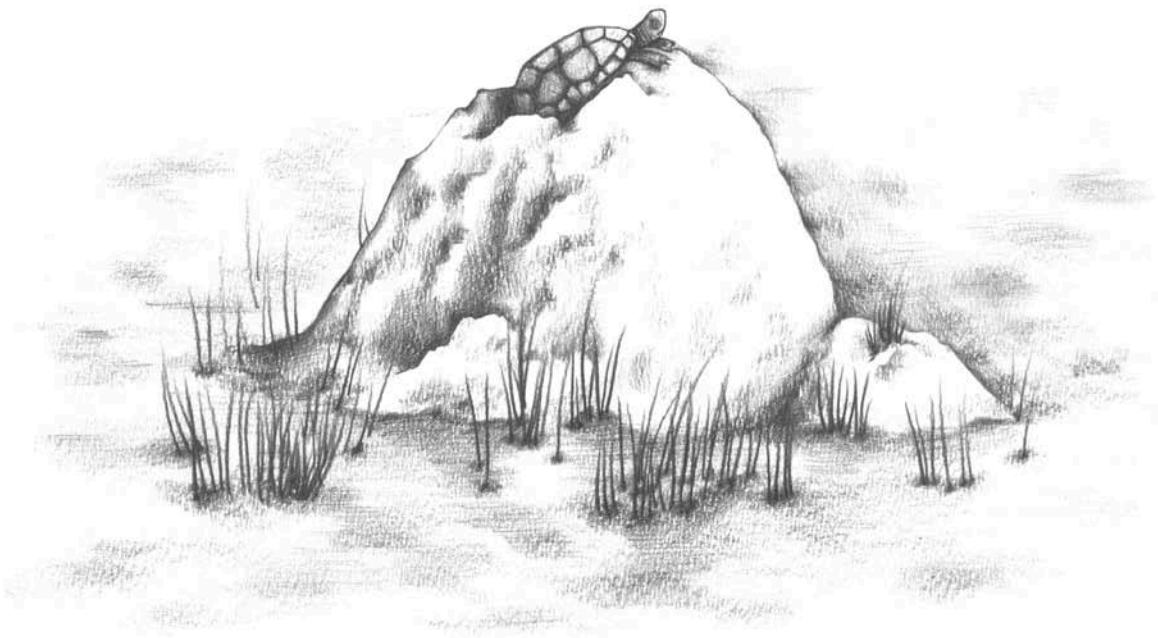
Chapter V



This chapter reproduces entirely the manuscript:

Ibáñez, A., López, P. & Martín, J. Reproductive state affects boldness under risk of predation but not exploratory activity of female Spanish terrapins. *Under review*.

Reproductive state affects boldness under risk of predation but not exploratory activity of female Spanish terrapins

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Abstract: During pregnancy, gravid females might diminish their locomotor performance due to the additional weight acquired. In addition, reproductive state of the females may also affect to other requirements such as thermoregulatory, metabolical and physiological, derived of egg production. Thus, boldness under risky situations should be optimized depending on the reproductive state of female turtles to avoid an encounter with a potential predator. Here, we examined how reproductive state influenced boldness under different risky contexts in female Spanish terrapins (*Mauremys leprosa*). We simulated predatory attacks of different risk level and measured the time that the turtles spent hiding entirely into their own shells (i.e. appearance times) and the subsequent time after emergence from the shell that the turtles spent immobile monitoring for predators before starting to escape actively (i.e. waiting times). We also performed a novel-environment test and measured the exploratory activity of the turtles. Results showed that, appearance, waiting and exploratory behaviour were not related among them. Thus, bold individual turtles when deciding the different phases of hiding behaviour were not necessarily bolder when exploring a new environment suggesting no evidence of behavioural syndrome in this species. In addition, only appearance times were affected by reproductive state of turtles. Thus, non-gravid females appeared relatively sooner from their shells after a predator attack. Similarly, gravid females carrying greater clutches tended to have longer appearance times. Thus, gravid females behaved shier and appeared relatively later from inside their shells than non-gravid ones suggesting that only larger clutches could affect boldness in risky contexts of Spanish terrapin females. However, the lack of difference in relative and absolute weight between gravid and non-gravid females suggest that differences in appearance times might be due to metabolic-physiological costs associated with egg production and embryo maintenance. In the same way, gravid females might be shier facing a predator due to their higher immediate fitness compared to non-gravid ones.

Key words: boldness, hiding behaviour, exploratory behaviour, predation risk, reproductive state

Introduction

Traditionally some studies have concerned on how to estimate the costs derived of reproduction (Renzick 1985, 1992). Costs of reproduction may occur in fecundity, survival or both (Bell 1980; Shine 1980; Brodie 1989; Landwer 1994). On the other hand, optimality models predict that prey with greater initial fitness should be more cautious against predators (Cooper & Frederick, 2007). This matches the "asset-protection principle" (Clark 1994) that predicts that individuals with lower fitness tend to take higher risks than those with higher fitness that take fewer risks, which would become asset protecting.

The tradeoff between current and future reproductive investment may produce a high cost for individuals (Reznick 1985; Roff 1992; Stearns 1992). In this way, current reproductive investment might represent a cost of reproduction expressed, for example, as diminished locomotor performance and lowered survivorship to the next clutch (Miles et al. 2000). Some studies have suggested that locomotor decreased capacities of gravid females could be due to the additional weight of the clutch (Shine 1980; Vandamme et al. 1989). Nevertheless, locomotor impairment of gravid females might be due to physiological changes associated to pregnancy rather than a direct effect of physical burden (Brodie 1989; Olsson et al. 2000). Gravid females may also shift their behaviour independently of an increased weight (Cooper et al. 1990; Schwarzkopf & Shine 1992). Along the same lines, because body temperatures before ovoposition influence developmental rates as well as phenotypic traits of the resultant offspring (Shine 2006), gravid females require basking at higher frequencies and for longer periods for reaching optimal temperatures for a correct embryo development (Braña 1993; Blázquez 1995; Brent Charland & Gregory 1995). On the other hand, energy

reserves are allocated during reproductive season of females and might be important in terms of fecundity (Doughty & Shine 1998). Metabolic rates tend to increase during gestation on pregnant female lizards due to embryogenesis (Angilletta & Sears 2000; Robert & Thompson 2000). In the same way, egg load occupies a great part of body cavity that might compress internal organs as lungs incurring on high energetic cost of breathing (Munns 2013). Thus, switching boldness in risky situations associated to gravidity in females might be also driven by thermoregulatory, metabolical and physiological requirements derived of egg production.

Exploratory behaviour and boldness have been defined like animal personality traits due to their individual consistency across the time (Sih 2004; Reale 07; Biro and Stamps 08). Personality traits may be context-dependent and could be partially explained by some biological traits (e.g. Sinn & Moltschaniwskyj 2005). Behavioural syndromes are suites of correlated behaviours reflecting individual consistency across multiple situations (Sih et al 2004). Along those lines, boldness under predation risk may covary with exploratory behaviour, thus supporting the existence of a behavioural syndrome. For example, in juvenile convict cichlids, *Amatitlania nigrofasciata*, faster explorers in a new environment were slower reactors when facing a predator (Jones and Godin 2010). Chaffinches (*Fringilla coelebs*) with higher activity level were more likely to freeze and had longer latency times to resume activity after the apparition of a model hawk (Quinn & Cresswell 2005). Also, there was a tendency between risk-taking behaviour and early exploratory behaviour in great tits (*Parus major*), where fast explores returned sooner to a feeding table than slow explorers (Van Oers et al. 2004).

Our main aim here was to test whether reproductive state of female terrapins *Mauremys leprosa* imposes a cost in their boldness (i.e. antipredatory hiding responses into their shells) and/or exploratory behaviour. This is a predominantly aquatic turtle

that remains most of the time submerged in water, but that needs to bask and nest in terrestrial habitats (Andreu & López-Jurado 1998). Turtles usually bask during long periods until they detect any potential predator and quickly dive into water reducing the probability to be captured (López et al. 2005). The main turtle predators of this species come from terrestrial environments, basically mammals and birds (Martín & López 1990). We simulated in the laboratory predatory attacks of different levels of risk and measured the hiding response of gravid and non gravid female turtles inside their shells. We also measured the exploratory activity of female turtles in a novel environment. Because these turtles may assess risk level and predator persistence after a simulated attack (Martín et al. 2005), we expected that gravid females should modulate their hiding behaviour after the predator attack by considering their higher costs, in comparison with non-gravid females, in terms of performance, thermoregulation and/or metabolism. In addition, gravid females have higher current reproductive value and then they should act more cautiously than non-gravid ones (Clark 1994). Thus, we predicted that gravid females should spent longer times hidden inside their shells until they decide to switch and escape actively to a safer refuge. In the same way, we also predicted that reproductive state should affect exploratory behaviour in a novel environment. We expected that gravid females should be shier or less explorative than non-gravid ones due to their lower degree of mobility and more constrained thermoregulation and metabolism. However, alternatively, we could expect the opposite pattern because the reduced manoeuvrability of gravid females could force them to increase their inspective behaviour to obtain detailed information of the surroundings and have a successful escape of possible predators (Frommen et al. 2009). Finally, due to the previous research supporting that both boldness and exploratory are a behavioural syndrome in

other taxa, we also predicted that more active exploratory individuals in a new environment should be bolder under predation risk situations.

Material and methods

Animals of study and husbandry

We captured 20 adult *M. leprosa* female turtles during spring 2011 in several small streams, ponds and tributaries of the Guadiana River located within dehesa oaklands (Alconchel and Olivenza, Badajoz province, SW Spain). We used a modified version of the traditional underwater funnel traps, which include a mesh chimney that reaches from the body of the trap to the surface allowing the turtles to come to the surface to breath (T & L Netmaking, Mooroolbark, Victoria, Australia; Kuchling 2003). We baited traps with sardines and protected them from terrestrial predators by waiting in the proximity. We revised the traps every hour to collect turtles. All collected turtles were alive and did not show any sign of being stressed inside the traps.

Turtles were transported in plastic cages (80 x 40 cm and 50 cm height) to "El Ventorrillo" Field Station, near Navacerrada (Madrid province) where experiments were conducted. The journey was made by car with duration of four hours while maintaining a constant temperature of 23 ºC to avoid heat stress. We did not observe any effect of transport on mortality or health state of the turtles. Turtles were housed in individual outdoor plastic aquaria (60 x 40 cm and 30 cm height) containing water and rocks that allow turtles to bask out of water and to protect of weather conditions. The photoperiod and temperature were the same as those of the surrounding area. Turtles were fed three times a week minced meat, earthworms, and a commercial compound of turtle "pellets".

Turtles were held in captivity and the investigator minimized contact with the animals before and during all behavioural experiments to avoid possible effects of habituation. All individuals were healthy and in good condition during the tests, and at the beginning of August, when the experiments were finished, turtles were returned to the exact locations of capture.

Reproductive state, size and body condition of females

In turtles, clutch size is directly obtainable from X-ray photographs and, thus, this technique has been effectively used to evaluate clutch size (Gibbons 1982; Gibbons et al. 1982; Iverson 1991). It allows reproductive data to be gathered in a non-destructive manner (Gibbons & Greene 1979). Thus, we used direct digital X-ray photography (Direct Digital System, SEDECAL) to determine gravity state of female turtles. Turtles were x-rayed on their abdomen, where was visible the presence or absence of eggs, on one plate at the same time (gravid: N = 7; non-gravid: N = 13; egg load of gravid females: $\overline{X} \pm 1SE = 8.1 \pm 0.6$ eggs, range = 6-10).

We used a metal ruler (1 mm precision) to measure carapace length (CL) as the greatest straight-line distance from the anterior end to the posterior end of the shell ($\overline{X} \pm 1SE = 190 \pm 3$ mm, range 168-209mm). We used a Pesola spring scale to measure body weight ($\overline{X} \pm 1SE = 952 \pm 40$ g, range = 680-1,300 g).

Hiding behaviour

We performed two different behavioural tests simulating predatory attacks with two different risk levels ("low" and "high"). Every female turtle (N = 20) was tested in all

treatments and the order of presentation was randomized. In the first treatment ("low risk"), we took one turtle from its home cage, handled it once, briefly (approx. 20 s) and gently, and then released it prone in the middle of an open field with short grass. For the "high risk" treatment, we took the turtle from their own aquaria and walked and tapped gently the turtle with the hand during 5 min, simulating that a predator had captured it, before releasing the turtle placed onto their carapace with the plastron upside in the grass field. To avoid other confounding effects that may affect risk perception of turtles (Burger & Gochfeld 1993; Cooper 1997; Cooper et al. 2003), the same person wearing the same clothing performed all tests following the same procedure. Turtles were used only once in each trial to avoid stress; the trials were spaced sufficiently (at least 1 day) so that fatigue resulting from one test did not affect subsequent tests. Before the trials, we allowed turtles to bask in their outdoor home cages for at least 2 h, which allowed them to attain and maintain an optimal body temperature within the activity and preferred temperature range of the species (Andreu & López-Jurado 1998; Keller & Busack 2001).

After the simulated attacks, the experimenter retreated and remained immobile observing with binoculars from a hidden position situated 5 m far from the turtle. After the simulated attack, and as a consequence of handling, turtles typically remained immobile and withdrawn entirely into the shell (i.e. the head, legs and tail were not or were only barely visible from above the carapace). We then measured the time that the turtle spent withdrawn into the shell, since we released it on the ground until the head emerged from the shell (i.e. when the eyes could be seen from above the shell; "appearance time"), and the time from appearance until the turtle emerged entirely from the shell and started to escape actively (i.e. when the turtle first touched the ground with the limbs or head) ("waiting times"). We chose these two times for posterior analyses

because “appearance times” represented a situation where the turtles had no visual information on the predator or the surrounding habitat (Martín et al. 2005). However, during “waiting times” turtles were able to check visually their surroundings and evaluate when to switch to an active escape (Martín et al. 2005).

Exploratory behaviour

We performed a novel-environment test in indoor conditions to assess the exploratory behaviour of the female turtles in a glass cage (100 x 50 x 50 cm), which surface was divided in six equally sized rectangle areas (33 x 25 cm). The cage contained a sand substrate, thus, simulating a terrestrial habitat which is subjected to higher risk than an aquatic one (Martín et al. 2005; Polo-Cavia et al. 2008). We gently took a turtle from its outdoor home cage, and released it on one of the corners of the experimental cage, always in the same position and orientation. The experimenter subsequently retreated and observed from a partially hidden position monitoring constantly the position of the turtle. Usually turtles spent some time remaining withdrawn into their shells until they put out the head and limbs and started to walk around the cage. Then, we measured the time spent until the turtle moved to another division of the cage (i.e. when crossed the divisor line for first time) (‘latency time’) and the total number of changes between divisions or the total number of crossed lines (‘activity’). Every trial had a maximum duration of 10 min. In a few cases (3 of 20 trials) the turtle did not move from the initial square during the total observation period and, then, we assigned 10 min for the variable “latency time” and zero for the variable “activity”.

Statistical analyses

To summarise the six variables describing boldness (i.e. hiding behaviour) under the two risk levels and exploratory behaviour of turtles we used a principal component analysis (PCA) with a Varimax rotation (Table 1). Then, we performed one-way analyses of covariance (ANCOVA) with the PC-scores values from this PCA as dependent variables, carapace length as a covariate and reproductive state (gravid vs non-gravid) of turtles as a fixed factor.

Results

Hiding and exploratory behaviour

The PCA for boldness measurements during hiding and exploratory behaviour produced three independent components that together accounted for 77 % of the variance (Table 1). The first PC (PC1) was correlated with exploratory behaviour in a novel environment; negatively with the variable describing longer times until the turtles initiated the first movement, and positively with the variable explaining a greater number of changes between divisions of the aquarium. Thus, PC1 described a gradient from shy turtles with longer latency times until the first movement and lower activity in a novel environment, to bold turtles with shorter latency times and higher exploratory activity. The second PC (PC2) was positively correlated with variables describing longer appearance times from inside the shell after a simulated attack in both “low risk” and “high risk” treatments. Thus, PC2 described a gradient from bold turtles that spent shorter times hidden into their shells to shy turtles that spent longer times hidden after a

simulated attack. The third PC (PC3) was related with waiting times across the two risk levels, positively with waiting times until they decided to escape under “low risk” and negatively with waiting times under “high risk”. Thus, PC3 described a gradient from bold turtles having shorter waiting times in the low risk treatment but longer waiting times under high risk.

The results of the PCA showed that there was a positive correlation between the appearance times during low risk treatment (when the turtle was prone) and during high risk treatment (Table 1). Thus, females that were bold with respect to time hidden in the shell after appearance in the low risk treatment were also bold in the high risk treatment. In contrast, turtles that were bold with respect to waiting times under low risk were shy under high risk and viceversa. Otherwise, with respect to exploratory behaviour, there was a negative correlation between the first time that a turtle crossed a line (latency time) and the total number of crossed lines (exploratory activity) in a novel environment (Table 1). Thus, bolder turtles initiated exploration earlier and subsequently had a higher activity level.

On the other hand, the separation of appearance, waiting and exploratory behaviour in different independent PCs indicated that these behaviours were not related among them. Thus, bold individual turtles when deciding the different phases of hiding behaviour were not necessarily bolder when exploring a new environment.

Table 1. Principal component analysis for the boldness of the turtles when hiding into their shells in response to simulated predator attacks under two risk levels and when exploring a novel environment

	PC1	PC2	PC3
Appearance time low risk	-0.19	**0.92**	-0.01
Appearance time high risk	0.21	**0.92**	0.07
Waiting time low risk	0.05	0.10	**0.68**
Waiting time high risk	0.01	0.06	**-0.81**
Latency time novel-environment	**-0.93**	-0.10	0.15
Activity novel-environment	**0.87**	-0.08	0.23
Eigenvalue	1.78	1.67	1.17
% Variance	29.7	27.8	19.5

Effect of females' reproductive state on hiding and exploratory behaviour

Gravid and non-gravid females did not significantly differ in carapace length (one way ANOVA: $F_{1,18} = 0.31$, $P = 0.58$; gravid: $\overline{X} \pm 1SE = 188 \pm 5$ mm; non-gravid = 191 ± 3 mm), or weight (one way ANOVA: $F_{1,18} = 0.71$, $P = 0.41$; gravid: $\overline{X} \pm 1SE = 905 \pm 19$ g; non-gravid= 977 ± 14 g). Similarly, reproductive state did not affect the relative weight of the females (ANCOVA with carapace length as covariate; $F_{1,17} = 1.18$, $P = 0.29$). Clutch size was significantly and positively related with body mass in gravid females (Spearman correlation, $r_s = 0.76$, $t_5 = 2.65$, $P = 0.046$).

Furthermore, results showed that some components of boldness of female turtles (described by the PC scores obtained from the PCA) were affected by reproductive

state. Thus, PC2 scores were significantly affected by reproductive state (one-way ANCOVA with carapace length as covariate; $F_{1,17} = 4.84$; $P = 0.04$; PC2 scores, gravid: $\overline{X} \pm 1SE = 0.53 \pm 0.33$; non-gravid: -0.29 ± 0.24). Thus, gravid turtles behave shier and had significantly longer appearance times from inside their shells (i.e. higher PC2 scores) than non-gravid females. In contrast, reproductive state did not affect waiting times (one-way ANCOVA with carapace length as covariate; PC3 : $F_{1,17} = 2.28$; $P = 0.15$; PC3 scores, gravid: $\overline{X} \pm 1SE = -0.45 \pm 0.37$; non-gravid: 0.24 + 0.27)

Neither exploratory behaviour was asignificanlty affected by reproductive state (one-way ANCOVA with carapace length as covariate; PC1: $F_{1,17} = 0.08$; $P = 0.78$; PC1 scores, gravid: mean $\pm$ 1SE=-0.09 ± 0.40; non-gravid: 0.05 ± 0.29).

Clutch size was not significantly related with exploratory behaviour of the turtles (Spearman correlation: PC1, $r_s = -0.05$, $t_5 = -0.12$, $P = 0.90$). Nevertheless, clutch size tended, although not significantly, to affect both appearance times (Spearman correlation: PC2; $r_s = 0.67$ $t_5 = 2.03$ $P = 0.09$) and waiting times (PC3; $r_s = 0.69$, $t_5 = 2.13$, $P = 0.08$) of gravid females.

Dicussion

Our results revealed three different independent axis of boldness in hiding and exploratory behaviour in a new environment for female Spanish terrapins. Thus, bolder turtles in a novel environment initiated their activity relatively faster and changed more often between parts of the aquaria. The second (PC2) and third PC (PC3) represent appearance and waiting times respectively, that are two components of the hiding behaviour of the turtles in response to a predatory attack. In agreement with a previous study (Ibáñez et al. Chapter IV), turtles typically spend more time withdrawn into their

shells until they appear but less subsequent time until they start to escape under high risk level. In agreement, under low risk level the pattern was the opposite: turtles spend longer waiting times and shorter appearance times (Ibáñez et al. Chapter IV). Thus, there is an increment of the time spent into the refuge (shell) but a reduction of the time when they decide to escape while risk increases because a low risk attack initially only requires a low intensity antipredatory response (i.e. short appearance time) but turtles might require longer times monitoring the surroundings due to the uncertainty provoked by a low-risk predator approach that could be followed of future successive attacks (Polo et al. 2011). However, while risk increases longer appearance times and shorter waiting times might be a more effective tactic to avoid an encounter with the predator because turtles should just confirm that the predator is not still ambushing in the area (Polo et al. 2011). In this way, appearance times represent the time till a turtle decide to emerge from a refuge where they do not have information of the surroundings (Martín et al. 2005). However, during waiting times turtles place out of the shell head and limbs and monitor for the surroundings increasing their probability of being detected by predators. Thus, bolder turtles spent shorter appearance in both risk levels. However, shy turtles during low risk level were bold when risk increased.

In our study, boldness in appearance and waiting times were not related to boldness in exploratory behaviour (i.e. independent axis represented by the PCs) of female terrapins. In contrast, individual differences in antipredatory and exploratory behaviour reflected a behavioural syndrome in some fish species. For example, in juvenile bluegill sunfish (*Lepomis macrochirus*), bolder individuals, were more active, more willing to inspect novel environments and more willing to inspect a potential predator and spend time in risky areas than shy individuals (Wilson & Godin 2009). In addition, juvenile convict cichlids, *Amatitlania nigrofasciata*, that were more

exploratory in a novel environment were slower to react to a simulated fish predator attack (Jones & Godin 2010). However, in one population of sticklebacks (*Gasterosteus aculeatus*) active explorers were also bold under predation risk but there was a lack of relation in other population of the same species (Bell 2005). The correlation across personality traits such as exploration in a novel-environment and boldness under risk seems to be driven by predation, and, thus when predation is strong both traits tends to covary (Bell 2005). In fact, the large size of female Spanish terrapins (Muñoz & Nicolau 2006) may render them difficult to capture by some of the potential predators in the region (Martín & López 1990). In agreement, in hatchling turtles the pattern of survivorship appeared to be result of size-dependent predation, suggesting a benefit for larger individuals (Janzen et al. 2000). In this context, the lack of correlation between activity and boldness in our study could be explained by the fact that bold females under risk could not necessarily need be more active in a new environment if the predator pressure is not strong.

Female turtles appearance times' during both "low" and "high" risk treatments were affected by their reproductive state, with gravid females having longer appearance times than non-gravid females. In addition, gravid females carrying greater clutches tended to appear relatively later from inside their shells than those carrying fewer eggs. . However, waiting times were not affected by female reproductive state, although gravid females with more eggs tended to have longer waiting times in "low" risk and shorter waiting times in "high" risk level. The differences in appearance times between gravid and non-gravid females might be attributed to the trade off between current and future reproductive effort. The current reproductive value of gravid females is much higher than non-gravid ones. The "asset-reproductive principle" predicts that the larger an individual's current reproductive value, the more important it

becomes that the asset be protected (Clark 1994). Thus, gravid females invested relatively more in current reproduction than non-gravid ones and should be more cautiously in front of a potential predator attack. On the other hand, gravidity may impose mechanical limitations for an active escape strategy due to weight of a clutch of eggs reduces their maximum running speed (Shine 1980; Cooper et al. 1990). However, similar to other study with lizards, in our study gravid and non-gravid females did not differ in weight although gravid females had less risky behaviour (Downes & Bauwens 2002). In other reptiles, such as sea turtles, females lipid reserves are maximal before breeding during vitellogenic process for follicular development, while plasma triglycerides decrease towards the end of nesting season suggesting that a decrease in energy resources is one of the main factors involved in the regulation of the reproduction (Hamann et al. 2002). Similarly, in the freshwater turtle *Emydura krefftii* a steady decline in the fat stores coincided with the period of follicular development in breeding females (Georges 1983).Thus, this decline in available triglycerides and fat stores associated with reproduction in gravid terrapins might compromise the energy available for their escape strategies. In addition, the pattern obtained in gravid females suggests that greater clutches (carried by large females) could also affect their appearance and waiting times after predatory simulations but those results must be taken carefully. In agreement, in female zebra finches an increase of egg production entailed a detriment in escape flight performance mediated through flight muscle loss rather than changes in body mass (Veasey et al. 2001). Similarly, gravid female turtles might be shier than non-gravid females under predation risk due to a detrainment in their physical condition associated to egg production rather than weight differences. Alternatively, higher bask requirements of gravid females could increase the probability of detection by predators (Downes & Bauwens 2002). In fact, turtles often

bask during long periods near water and escape diving to water before they could be visually detected by the observer (López et al. 2005). In addition, the rate of consumption of oxygen tends to increase with the number of embryos carried by the female suggesting that the metabolic costs increases according with the number of eggs (Birchard et al. 1984). Thus, gravid turtles with more eggs could need to bask for longer periods as happens in gravid lizards (Shine 1980). In this way, gravid females with greater clutches could need longer appearance times and waiting times (in low risk) to reach an optimal temperature due to the higher requirements of egg production. Paradoxically, females carrying more eggs tended to have shorter waiting times under high risk probably because in this context the turtle have assumed that have been detected by the predator rendering unimportant other traits.

Otherwise, explorative behaviour was not affected by reproductive state of the female turtles. Exploratory behaviour has been shown as hereditable and advantageous in some contexts, for example, in great tits (*Parus major*), faster exploratory parents had offspring with greater ability for disperse in wild habitats (Dingemanse et al. 2003). Similarly, more exploratory lizards in a novel environment should enhance fast risk assessment abilities (Rodríguez-Prieto et al. 2011). However in our study, gravidity did not affect boldness in exploratory behaviour suggesting that this factor might be unimportant for exploration in new environments or that the effect might be masked by other factors.

In conclusion, hiding and exploratory behaviour were not related providing evidence that both traits are not a behavioural syndrome in female of Spanish terrapins. The results obtained highlighted that under risky situations, some components of boldness (i.e. appearance times; PC2) were affected by reproductive state of females. However, other components of boldness such as exploratory behaviour (PC1) or

waiting times (BPC3) did not differ between reproductive state classes. Gravid females appeared relatively later from into their shells after predatory attack respect to non-gravid ones. In addition, gravid females carrying a greater number of eggs tended, although not significantly, to have longer appearance times as well as longer waiting times during "low" risk but shorter waiting times in "high" risk treatment. Those results may suggest that larger clutches could affect boldness in risky contexts of Spanish terrapin females. The lack of difference in relative and absolute weight between gravid and non-gravid females suggest that differences in appearance times might be due to metabolic-physiological costs and a worse condition of gravid females associated with egg production and embryo maintain. However, gravid females might act more cautiously due to protect their immediate higher fitness to respect to non-gravid ones. Indeed, further studies should clarify the evolution of the behavioural strategies in risky contexts and the relation with female reproductive state.

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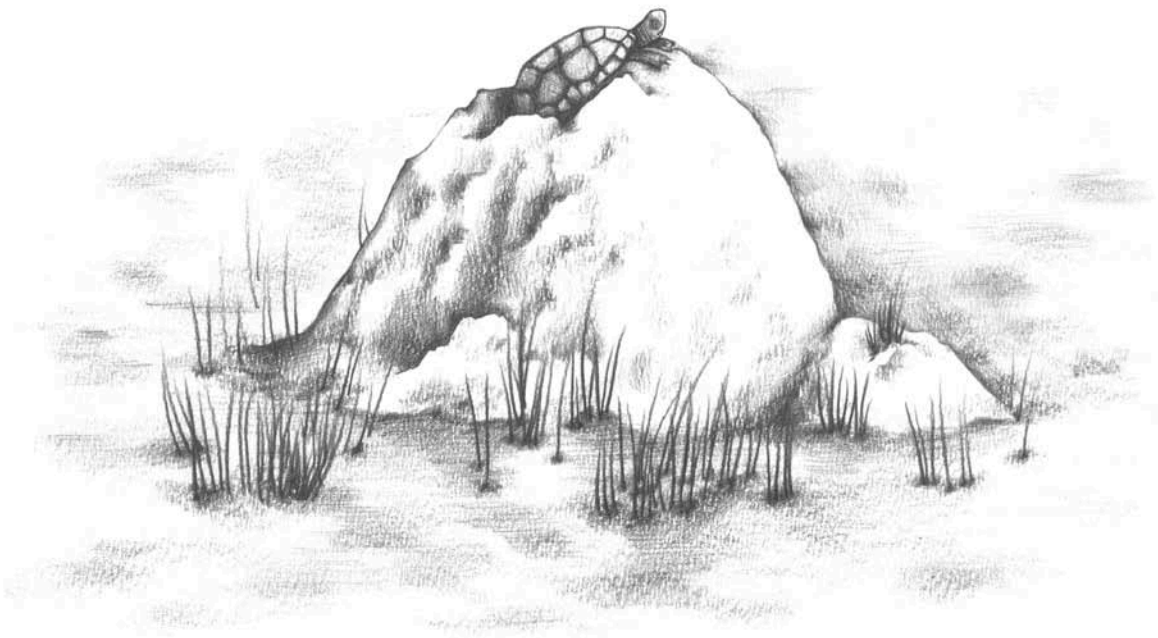
Chapter VI



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Basking behaviour is modulated by health state and colour conspicuity to predators in a wild population of Spanish terrapins

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Abstract: Aerial basking may have several benefits for freshwater turtles in addition to thermoregulation such as removing parasites from the skin, which would improve health state. However, basking outside of water may be risky because it may expose freshwater turtles to terrestrial predators. Here, we monitored the basking activity of male Spanish terrapins (*Mauremys leprosa*) in a wild population of the south-western of the Iberian Peninsula. We also measured body size, health state parameters and limb coloration of these individuals. We aimed to examine whether basking may improve health state of turtles by examining the relationship between basking activity and health state. Contrary to our expectations, the results showed that male turtles with higher basking activity had lower total white blood cell (WBC) counts and lower values of haematocrit. This might indicate that during basking turtles are more exposed to infections or that they spend less time foraging compromising their immune system. In addition, because basking might be risky we also expected that those turtles with more conspicuous coloration should reduce their basking activity. We found that male turtles that spent more time basking also had less bright coloration in limb stripes. Thus, showier turtles (those with brighter limb stripes) may reduce their basking activity to avoid being detected by potential predators. Our study provides evidence that basking activity may have several costs in terms of health state and predation for the turtles. Understanding the balance between the benefits and the costs of basking might be essential for the conservation of freshwater turtle populations.

Keywords: Basking, health state, coloration, haematocrit, total WBC counts, predation risk

Introduction

Benefits of basking behaviour in reptiles have been mainly attributed to thermoregulation with less importance in terms of general health of the epidermis and on reducing the incidence of plant and animal parasites (Boyer 1965). In addition, an increase of aerial basking activity in freshwater turtles may promote digestion (Cagle 1950; Moll & Legler 1971; Spray & May 1972), enhance the synthesis of Vitamin D in the skin (Acierno et al. 2006; Pritchard & Greenhood 1968) and remove algae and parasites from the shell and skin (Boyer 1965; Cagle 1950; Neil & Allen 1954). However, an increase of basking activity outside of water might increase predation risk by terrestrial predators, thus, reducing survival probability, which suggests the existence of a potential trade-off between the benefits and the risks of aerial basking behaviour in freshwater turtles.

Basking outside of water has associated several benefits. As in other reptiles, for freshwater turtles basking might be essential to reach an optimal temperature necessary for vital activities. However, metabolic rate is positively related with body size in endotherms and ectotherms (Gillooly et al. 2001). Also, turtle body weight is the most important biological factor affecting heating rates during basking, with smaller turtles warming faster than larger ones (Boyer 1965; Polo-Cavia et al. 2009). Thus, larger turtles should bask during longer periods to reach their optimal temperature and afford their higher metabolical expenditures.

Also, with respect to the benefits of basking for eliminating parasites, previous research showed that the surface of carapace of chelonians might be colonized by certain epizootic organisms such as algae (Burgin & Renshaw 2008; Colt et al. 1995; Edgreen et al. 1953; Garbary et al. 2007; Soylu et al. 2006), protozoa (Bovee 1976) and

mollusks (Frazier et al. 1985). However, the relationships between turtles and epizootic organisms living on them are not clear and it has been speculated that might be evolved as either symbionts or parasites. On the other hand, leeches are common ectoparasites of freshwater turtles (McCoy et al. 2007; Ryan & Lambert 2005; Siddall & Gaffney 2009; Watermolen 1996) that may transmit haemogregarine blood parasites specific of chelonian hosts (Siddall & Desser 2001). Several studies have shown that bottom-dwelling species of turtles have higher leech load that those which bask aerially (McCoy et al. 2007; Readel et al. 2008; Ryan & Lambert 2005). One hypothesis to explain this differences across turtle species in their leech load is the "desiccation hypothesis" that suggests that aerial basking is the proximate cause to encourage to leeches detaching from the basking turtles to avoid desiccation (Ernst 1971; MacCulloch 1981; McAuliffe 1977). However, Ryan and Lambert (2005) found higher colonization rate by leeches in bottom-dwelling turtles than in aerial basking ones even when both species were not allowed to bask, suggesting that there are other factors apart of the "desiccation hypothesis" involved in leech infestation on freshwater turtles.

However, a theoretical model of cost-benefits predicts that thermoregulation is benefitial only when costs are low (Huey & Slatkin 1976). One of the main costs incurring by reptiles when basking, for either thermoregulating or eliminating parasites, can be predation (Pianka & Pianka 1970; Huey 1982; Carrascal et al. 1992). In fact, in some areas, the main predators of freshwater turtles inhabit terrestrial environments (Martín & López 1990) while water bodies might represent safer habitats with lower predation rate. Survivorship of wild painted turtles (*Chrysemys picta*) depends of environmental factors such as the number of available safe basking sites (Wilbur 1975). Similarly, the Murray turtle (*Emydura macquarii*) may bask in aquatic environments with similar benefits than aerial basking but with fewer costs in terms of predation risk

and feeding opportunities (Chessman 1987). Thus, while basking, turtles may assume the risk of facing predators. However, turtles may reduce the probability of being captured by diving to the water when they detect any approaching potential predator (López et al. 2005), although this has the cost of interrumpting basking. In this context, the degree of showiness (i.e. coloration) of the terrapins may compromise the risk of being detected while basking by bird predators (Honkavaara et al. 2002; Viitala et al. 1995).

In previous studies, we found that male Spanish terrapins, *Mauremys leprosa* with more conspicuous limb stripe coloration spend longer times hidden inside the shell after a predator attack, suggesting that more conspicuous individuals may act more cautiously due to their higher probability of being detected by predators (Ibáñez et al. Chapter V). In this study, we examined in the field the relation of basking patterns of male terrapins, *M. leprosa*, with health state, body size and limb colour showiness. Because basking behaviour might have important benefits in terms of parasite skin protection, we expected that those individuals with higher basking activity should have better health state than those with lower basking activity. In addition, larger turtles should need longer basking times to afford their slower heating rates. However, basking behaviour might be risky because the main predators in the study site come from terrestrial habitats (Martín & López 1990). Thus, we predicted that turtles with greater skin color conspicuity might be more easily detectable by potential predators while basking, and should, therefore, reduce basking activity to decrease predation risk.

Material and methods

Anials of study

During early spring 2011 as a part of a long-term mark-recapture study we trapped turtles in several ponds and tributaries of the Guadiana River close to Olivenza and Alconchel (Badajoz province, southwestern Spain). We used a modified version of the traditional underwater funnel traps, which include a mesh chimney that reaches from the body of the trap to the surface allowing the turtles to come to the surface to breath (Kuchling, 2003; T & L Netmaking, Mooroolbark, Victoria, Australia). These freshwater habitats, located in dehesa woodland, contain a large population of turtles. After capturing the turtles, we studied the basking activity pattern of 27 adult male Spanish terrapins in two different ponds situated close to Alconchel. As we captured more males than females at the beginning of the mating season (see Ibáñez et al. 2013), we could only mark and monitor the basking activity of three females (not included in the analyses; see below).

Turtles were held in captivity in a country house building close to the capture sites while we marked them and took blood samples and morphological, and coloration measurements. The turtles were kept in captivity the shortest time possible (approximately two weeks) to avoid any effect in their behaviour and health state. Turtles were housed in individual outdoor plastic aquaria (60x40x30 cm) containing water and rocks that allowed turtles to get out of water. Turtles were regularly fed with minced meat, earthworms, and a commercial compound of fish "pellets". Photoperiod and temperature were the same as those of the surrounding area. Once all measurements were collected, turtles were returned to the exact locations of capture in good health.

Size and physiological-health measurements

We measured turtle size (maximum straight carapace length) and body mass the same day of capture with a metal ruler and a Pesola respectively. We also calculated body condition of individuals as the residuals of the regression between log-transformed weight and body length (Polo-Cavia et al. 2010). Blood samples were collected from the caudal sinus at the base of the tail by using a 1 mL syringe, with a 30 G, 1/2, 0.3 x 13 mm heparinized needle for each turtle (Polo-Cavia et al. 2010). Blood was placed in a heparinized capillary tube and centrifuged during 10 min at 10,000 rpm. The haematocrit (i.e., the volume percentage of red blood cells in blood) was measured with a graphical scale in each capillary tube as the ratio between the length of the capillary tube occupied by packed red blood cells and the total length of the capillary tube occupied by the blood sample. Because the haematocrit was significantly and negatively related with body mass ($r=-0.46$ $N=27$, $F_{1,25}=6.72$, $P=0.016$), we calculated the residuals of the regression between haematocrit and body mass to test the effects of the haematocrit independently of turtle size. We also prepared a smear on a microscope slide from the blood taken. Blood smears were air-dried, fixed in absolute ethanol for 10 min, and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) for 40 min before their examination. Blood smears were used to estimate the total numbers of white blood cells (WBC) and the proportions of different types of leucocytes under magnification, ×1000. The proportions of different types of leucocytes were assessed on the basis of an examination of a total of 100 leucocytes. The number was quantified as number of leucocytes per 100 fields examined. The heterophile:lymphocyte (H:L) ratio

was estimated from the percentages of heterophiles and lymphocytes per 100 leucocytes obtained in these counts (Merino et al. 1999).

To summarize the six variables describing size and physiological-health measurements of the turtles, we used a principal component analysis (PCA) with a Varimax rotation (see results and Table 1). Health-PCscores resulting from this PCA were used in further analyses.

Colour measurements

Reflectance spectra of turtles' limb stripes between 300 and 700 nm were recorded using an Ocean Optics UV VIS (JAZ-EL200) spectrophotometer (Ocean Optics, Inc., Dunedin, FL, USA) . Mean reflectance was summarized over 5 nm steps ("binned", Grill & Rush 2000). Turtles had apparently uniform colour in limb and neck stripes. However, turtles have retractable necks and usually hid the head inside the shell when handled. Thus, due to the difficulty of measuring coloration in the neck stripes of turtles and to prevent harming them, we measured coloration at five random places of the forelimbs orange stripes (right and left). Then, we calculated a mean for the limbs and used these values for further analyses. Otherwise, even shell the most visible part of the turtles while they are basking we did not measure carapace coloration due to the cryptic role in this species (Ibáñez et al. 2013). Reflection was recorded using a probe held normal to the surface, and reflectance was always measured by the same person (AI). A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

We mathematically summarized the spectra using principal component analysis (PCA) (Cuthill et al. 1999; Endler 1990; Grill & Rush 2000). The PCA summarizes all

of information about the shape of complex reflectance spectra, including bimodal ones like those that we measured in our turtle species (Ibáñez et al. 2013), into a few PCs that are independent of one another (Montgomerie 2006). In PCA of spectral data, PC1 represents variation in intensity of coloration or brightness, and subsequent PCs represent combinations of hue and chroma (Cuthill et al. 1999; Endler 1990; Grill & Rush 2000). Also, the PCA identifies those sections of the spectrum (wavelength regions) that are contributing to the observed variation, independently of their "importance" in terms of contribution to the total amount of reflectance (Montgomerie 2006). The scores from the PCA on limb stripe reflectance of the turtles were also used for further analysis.

Basking behaviour of turtles

We used plastic labels with alphanumeric codes for long-distance detection and individual identification. To mark the turtles on the dorsal part of the shell with the plastic labels, we first used sandpaper to remove irregularities of the second vertebral shell scute and, then, we fasten the plastic label with epoxy glue to the scute. Subsequently, turtles were placed in individual plastic boxes and left there overnight. The day after, turtles were released to their origin ponds for monitoring their behaviour during the next months. We checked the behaviour of the turtles using binoculars and a telescope watching from a hidden position near the pond.

The same person (AI) monitored turtles basking activity during 19 days divided between the months of April and May. The observations were made between 10.00 AM and 19.30 PM covering a wide-range of freshwater turtle daily activity periods (Rowe & Moll 1991). We made observation in different periods of the day that ranged between

20 and 290 min. In every period of observation we tried to identify the maximum number of turtles through the plastic labels. Then, we monitored the behaviour of each turtle identified till the end of the period or till it was not visible anymore (i.e. dive into water). We considered that a turtle was basking when it was remaining immobile out of the water, exposed to solar radiation directly or solar heated surfaces, such as shorelines, rocks or deadwood logs. Thus, we noted down the time devoted to basking by each turtle. Then, we determined a specific basking activity index for each individual, expressed as the ratio between the basking time of the turtle and the overall monitoring time for the pond.

Statistical analyses

First, we ran a backward stepwise general regression model (GRM) to explore the possible relation between health-state and limb coloration of the turtles with the health-PC scores as dependent variables and the colour-PC scores as independent variables. We also calculated the square root of basking activity index to normalize this data (Shapiro-Wilk test; $P > 0.50$). Then, we used a backward stepwise general regression model (GRM) to examine the relationships between basking activity (dependent variable) with turtle traits (i.e. body size and physiological-health parameters) summarized by the health-PC-scores values and coloration of the limbs described by the colour-PC-scores as independent variables.

Results

Size and physiological-health measurements

The PCA for size and physiological-health measurements produced three independent components with eigenvalues greater than one, which together accounted for 82.5% of the variance (Table 1). The first PC (health-PC1) was positively correlated with variables describing absolute size of the turtles. Thus, health-PC1 described a gradient from smaller and lighter turtles to larger and heavier turtles. The second PC (health-PC2) was positively correlated with variables describing total WBC counts and the residuals of haematocrit values independently of body size. Thus, health-PC2 described a gradient from turtles with lower leukocyte count and haematocrit values to turtles with higher leukocytes count and haematocrit values. The third PC (health-PC3) was positively related with H:L ratio and relative size (body condition) of the turtles. Thus, health-PC3 described a gradient from turtles having lower H:L ratio and lower body condition to higher H:L ratio and higher body condition.

***Table 1**. Principal component analysis (MPC) for the body size and physiological-health measurements of the turtles*

	health-PC1	health-PC2	health-PC3
Carapace length	**0.99**	-0.02	-0.02
Body mass	**0.90**	-0.08	0.40
Body condition	0.06	-0.12	**0.86**
Total leukocytes (WBC)	-0.15	**0.88**	0.40
Haematocrit residuals	0.06	**0.88**	-0.15
H:L ratio	0.16	0.05	**0.77**
Eigenvalue	2.28	1.50	1.16
% Variance	38.0	25.1	19.4

Limb coloration

The PCA for coloration of the limb stripes produced three principal components. Pattern of coefficients relating color-PC1 (eigenvalue = 58; 71.6 % of variance explained) to the original reflectance data were all negative and of similar magnitude and relatively flat across the spectra of all individuals, so colour-PC1 represented achromatic brightness variation in original spectra, with turtles with lower colour-PC1 scores having brighter limb coloration. The colour-PC2 (eigenvalue = 15.6; 19.2 % of variance) and color-PC3 (eigenvalue = 5.8; 7.2 % of variance) were not spectrally flat

and thus represented variation (hue and saturation) of the limbs. The coefficients relating colour-PC2 to the original reflectance values were high and positive below 500 nm and were negative above 500 nm. Thus, colour-PC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower colour-PC2 scores indicating limb stripes with more saturated orange coloration. Pattern of coefficients of colour-PC3 suggested it represented variation in the relative amounts of medium (400-580 nm) wavelengths in the positive side to both short (300-400 nm) and long (580-700 nm) wavelengths in the negative side. We used the scores of the PCA for further analyses.

Relationship between health state and limb coloration

Health-PC1 was not significantly related with any PC describing limb coloration (stepwise GRM, all $P > 0.18$, i.e. not included in the final model). Similarly, health-PC2 did not significantly depended of any variable initially included in the model (stepwise GRM, all $P > 0.51$, i.e. not included in the final model). Neither health-PC3 was asignificantly affected by limb coloration (stepwise GRM, all $P > 0.36$, i.e. not included in the final model).

Relationship between basking activity, size, physiological-health state and coloration

Males' basking activity was significantly and negatively related with health-PC2 and significantly and positively related with colour-PC1 (stepwise GRM, model: $R^2 = 0.25$; $F_{2,24} = 5.35$; $P = 0.01$; health-PC2: $\beta = -0.36$; $t = -2.15$; $P = 0.04$; colour-PC1: $\beta = 0.38$; $t = 2.23$; $P = 0.03$; Fig. 1), the other variables were not significant and were excluded

from the final model. Thus, males that spent more time basking had lower values of total WBC counts and haematocrit than those that spent less time basking. In addition, males with high basking activity had less bright limb stripes.

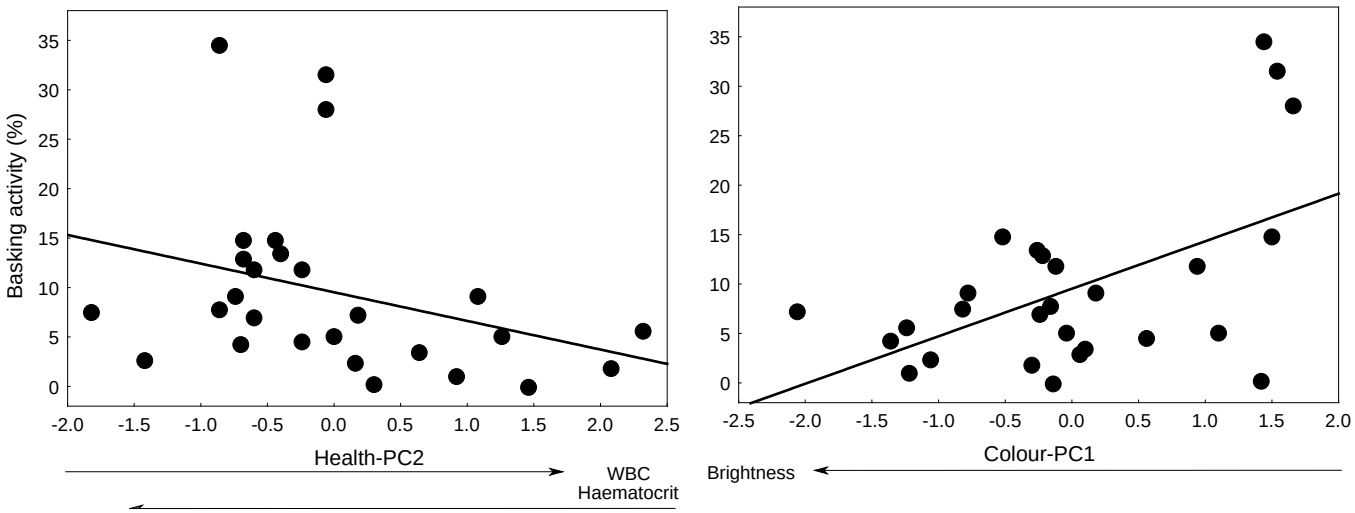

Figure 1. Relationship between basking activity and health-PC2 (left) and brightness of the limb stripes (colour-PC1) (right). Arrow indicates the relationship of WBC and haematocrit with health-MPC2 (left) and brightness of coloration with colour-PC1.

Discussion

Our results showed that basking activity was related with health state and colour showiness of the limb stripes in male Spanish terrapins. However, contrary to our predictions, body size did not influence basking activity of the turtles.

Concerning the health state of the Spanish terrapins, some physiological-health variables were correlated. Firstly, males having more total WBC counts also had higher values of haematocrit. In *Mauremys leprosa* the highest percentages of blood lymphocytes were obtained in spring suggesting that white blood cells are more abundant during periods of more activity, when turtles are more exposed to infection by

pathogens (Muñoz & De la Fuente, 2004). Similarly, low haematocrit values have been related with sickness and malnutrition in freshwater turtles (Knotkova et al., 2005; Tavares-Dias et al. 2009). Also, heterophil to lymphocyte ratio was positively related with body condition in our study. H:L ratio have been proposed as a reliable indicator of long-term stress in reptiles (Mader 2000; Davis et al. 2008). For example, western pond turtles (*Emys marmorata*) from altered habitats had higher body condition and H:L ratio compared with those turtles living less disturbed habitats (Polo-Cavia et al. 2010). Otherwise, body condition calculated as the residuals between body mass and carapace length might not reflect a real estimation of body fat stores as a lot of variation is due to shell weight and might not be a reliable indicator of immunological state of the turtles (Polo-Cavia et al. 2010).

Basking activity was not related to body size in spite that larger turtles require longer basking times to reach a temperature optimal for normal activity (Boyer 1965; Cloudsley-Thompson 1971; Hutchison & Maness 1979; Huey 1982, Polo-Cavia et al. 2009). However, different studies obtained contradictory results in the relation between basking activity and body size in freshwater turtles. For example, larger male painted turtles (*Chrysemys picta*) basked during longer periods of time in one study (Carrière et al. 2008), but, in other study, there was no relation between body size and the length of basking bout among adults (Lefevre & Brooks 1995). In agreement with the latter, we found that basking activity of larger and smaller male *M. leprosa* individuals was similar. In our study all males were adult and the range of variation in size of the individuals was limited. This fact, might induce that turtles have similar metabolic demand and consequently the same basking requirements. Similarly, the differences in the heating rates between relatively smaller and larger males might be very small making the body size of the turtles irrelevant in our study.

Basking activity was related with some variables that can reliable indicate the physiological-health state of the turtles, such as haematocrit and total WBC count. For example, erythrocytes have a pivotal role in tissue oxygenation. An increased haematocrit might be an adaptation for air-breathing vertebrates that remain diving for long periods in aquatic habitats. For example, sea turtles have the highest values of haematocrit in reptiles and are comparables to the values found in marine mammals (Lutcavage & Lutz 1997). Thus, a higher value of haematocrit might provide a higher aerobic capacity improving locomotor performance and mobility in male terrapins, as happens in the lizard *Amphibolurus nuchalis* (Garland & Else 1987). In addition, leukocyte profiles can provide a reliable assessment of stress in vertebrates (reviewed in Davies et al. 2008). In the case of H:L ratio, it may be used as a compliment to the measurement of adrenal hormones in the study of vertebrate stress responses (Davies et al. 2008). For example, individuals of *E. marmorata* living in a wastewater treatment plant, subjected to a high level of pollution, had higher H:L ratio than those turtles living in an ecological reserve (Polo-Cavia et al. 2010). However, in our study basking activity was not related with the H:L of the turtles. On the other hand, the relation of total WBC counts with individual stress seems inconsistent across the literature (reviewed in Davis et al. 2008). Infections commonly cause general increases in total WBC counts in vertebrates (Davis et al. 2008). For example, chickens that had an acute inflammation experienced a significant increase of leukocytes persistent through seven days (Latimer et al. 1988). However, it is not clear whether higher total WBC counts reflect a better or a worse immune system. For example, offspring of ornamented male three-spined sticklebacks, *Gasterosteus aculeatus,* have higher WBC counts, which are interpreted as a greater parasite resistance inherited from their parents (Barber et al. 2001). In freshwater turtles, both total white and red cells counts have been evaluated

together in some studies suggesting their dependence on ecological conditions and individual nutritional state. In freshwater turtles of the species *Emys orbicularis* and *Mauremys rivulata*, blood cell counts depended on habitat quality with an increase of erythrocytes and leukocytes in contaminated water (Tosunoglu 2011). The authors argued that the high leukocyte values are due to water pollution, while high erythrocytes values were caused by decrease in oxygen (Tosunoglu 2011). However, western pond turtles from contaminated habitats had lower immune response than those from non-contaminated habitats but the total WBC counts did not depend on the habitat alteration (Polo-Cavia et al. 2010). In addition, malnourished giant Amazon turtles (*Podocnemis expansa*) had lower values of haematocrit and lower total WBC counts than turtles fed in normal conditions (Tavares-Dias et al. 2009). Thus, a possible explanation for the low haematocrit values observed in the terrapins with high basking activity might be that they had more static lifestyle and consequently a reduced oxygen demand. Similarly, male terrapins that dedicate more time to basking might reduce the time spent in other activities, such as foraging, compromising the amount of energy invested in their immune system, and consequently have lower total WBC counts. In any case, our results seem to indicate that an increased basking activity might compromise the health state of male turtles.

Different components of coloration may reflect several phenotypic traits in several reptiles (Weis 2006; 2011; Martín and López 2009). Showy coloration in males of several species may be favored through sexual selection (Andersson 1994). In birds, predation risk is likely to influence the evolution of coloration, disfavouring bright coloration in some species (Götmark 1993). In freshwater turtles, shell is the most visible part exposed to predators during basking activity. In fact, shell colour variation across different habitat types seems correlated with substrate ground coloration (Rowe

et al. 2006a; McGaugh 2008). Previous studies with different species of freshwater turtles have suggested that shell coloration might be involved in crypsis (Rowe et al. 2013; Ibáñez et al. 2013). Thus, we did not consider shell coloration for our study. Otherwise, the head and limbs of turtles can be hidden, at least partially, by retracting them inside the shell and are not always exposed to predators. Thus, limb coloration can be more easily subjected to sexual selection forces and withstand an exaggerated ornamentation than shell coloration (Ibáñez et al. 2013). Thereby, terrapins having brighter limb stripes might be more conspicuous for predatory birds. In line with those results, males with brighter coloration in their limb stripes behave shier after a simulated predatory attack and have longer hiding times inside their shells (Ibáñez et al. Chapter V). In freshwater turtles, aerial basking in terrestrial habitats may be risky. For example, wood turtles are able to regulate their body temperature through basking behaviour, but thermoregulation is imprecise suggesting that predation risk could constrain basking behaviour while turtles are exposed on bare ground (Dubois et al. 2009). Thus, turtles having striking coloration (i.e. brighter skin in limb stripes) should assume higher risks during basking activity than those having less striking colours. In this context, more conspicuous individuals may reduce basking times to reduce the probability of being detected and captured by potential predators.

However our results must be taken carefully because basking activity of female terrapins was not monitored in the present study. In *M. leprosa*, females have more conspicuous colorations (i.e. brighter and more orange saturated) in their limb stripes than males (Ibáñez et al. 2013). Also, traits that may reflect phenotypical quality (i.e. body size or immunocompetence) are related with coloration of limb stripes in females but not in males (Ibáñez et al. 2013). This raises the question of whether female turtles should be more careful during basking behaviour due to their higher conspicuity to

predator eyes. However, limb coloration was an unimportant variable determining antipredator hiding behaviour in females suggesting that other factors may influence predation risk in female *M. leprosa* (Ibáñez et al. Chapter V). It is likely that the larger size of females in comparison with males might make females more difficult to be captured by predators, which would make interindividual coloration differences less important in females. The study of basking behaviour in female turtles would have allowed us to clearly disentangle whether limb coloration affects their patterns of basking in the wild as might happen with male turtles.

In conclusion, basking activity was related with health state (i.e. total number of leukocytes and haematocrit) and limb coloration. Male turtles that spent less time basking had higher total WBC counts and higher haematocrit values. Thus, males with higher basking activity could have less oxygen requirements due to their lower mobility and consequently reduced metabolical expenditure. Similarly, males that spent more time basking could be more susceptible to infection due to the fact that they dedicate less time to foraging which may compromise their immune system. Otherwise, basking behaviour could carry associate costs in terms of predation risk with more conspicuous individuals reducing their basking activity to avoid be easily detected by potential predators. Further studies should include females to clarify the role of coloration showiness in basking patterns in freshwater turtles.

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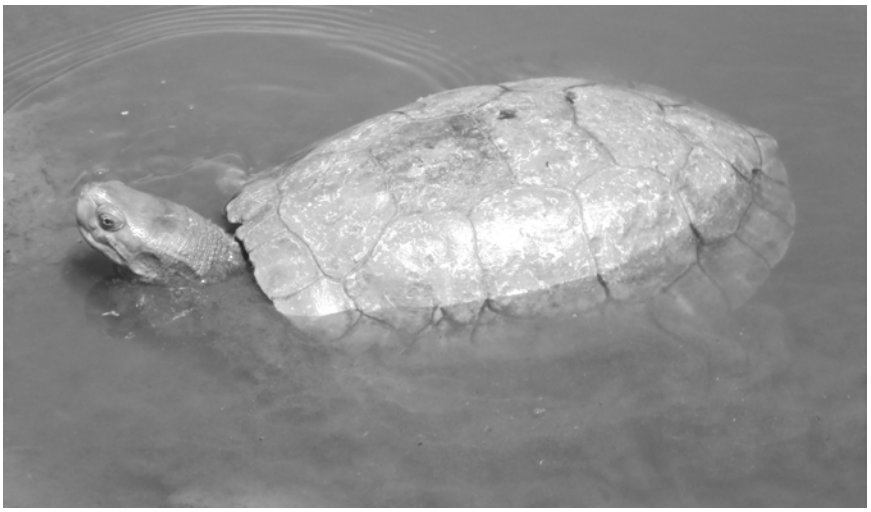
Abstract



Introduction

To communicate animals may use a wide range of sensory modalities such as tactile, chemical, visual and auditory systems.

In reptiles, on the one hand, intraspecific chemical communication has a crucial role in mate searching and mate selection, as well as in discrimination of the quality of potential partners. Chemical signals are also important in intrasexual relationships between males, where they can contribute to reduce costs of aggressive interactions. Many studies have demonstrated that turtles have very well-developed nasal and vomeronasal systems that are mainly used to make discriminations of different scents, suggesting a good ability of turtles to detect chemical cues in the environment. In agreement, recent laboratory experiments have showed the importance of inter-and intrasexual chemical communication in turtles.

On the other hand, reptiles display conspicuous colorations during the breeding season that is also important for partner selection and in agonistic interactions between males. Several studies have demonstrated the existence of sexual dichromatic coloration in some turtles. However, only a few recent studies have shown that colourful skin patches and stripes of the head of turtles may be linked with individual quality, suggesting that coloration could evolve under sexual selection pressures in turtles.

Sexual signals are directed to find a mate with the benefit of reproduction, enhancing the fitness of the sender. However, during sexual signalling, the individuals are also more vulnerable to be localized by enemies due to their greater detectability intended to receivers. In this framework, signals addressed to conspecifics for inter- and intrasexual communication may be exploited by predators. In addition, reproductive

females may be more exposed to predators during the breeding season due to behavioural and physiological changes related with reproduction. Similarly, gravid females may show decreased locomotor performance that might compromise their escape strategies when facing a predator. However, in spite of increased predation risk, individuals with extravagant ornamentation, as well as those in a gravid reproductive condition, might behaviourally compensate by acting more prudently, thus, reducing predation risk.

In temperate regions, the main hazard for freshwater turtles comes from terrestrial predators, basically mammals and birds. In this context, turtles have morphological structures such as the shell that offers partial protection (i.e. refuge) when they are in terrestrial habitats, until they can escape to a safer refuge deep in the water. Therefore, turtles can withdrawn and hide the legs, head and tail inside the shell when a predator approaches too close and/or try to attack them. However, increasing the time spent hiding in a refuge, or into the shell, may entail a loss of feedingand mating opportunities or thermoregulatory costs. Therefore, the decision of when to emerge from the refuge and initiate an active escape may lead to a trade-off that animals should be able to optimize by modifying hiding times. Hiding times tend to increase with the costs of emerging attributable to predation risk, suggesting that the ability to assess the risk level is essential to decide when to leave the refuge. The balance between the costs of remaining hidden vs. costs of emergence should also depend on several traits such as body size, conspicuousness of sexual coloration, reproductive state or health state. These factors may explain the inter-individual variations in boldness under predation risks, which have often been used to define different personality types. In fact, natural selection, through the predation regime, may be one of the main forces modulating the evolution of personality traits.

Objectives

Chapter I. This study examined the behavioural responses of Spanish terrapins (*Mauremys leprosa*) to chemical cues of conspecific individuals of different body size and health state. The first experiment aimed to examine the potential role of chemical cues in partner choice. In a second experiment, we examined the behavioural responses of males to chemical cues of other males.

Chapter II. This study examined whether male *M. leprosa* turtles were able to recognise and distinguish familiar from unfamiliar males via chemical cues released to water, and the influence of body size and boldness of male turtles on their response to chemical cues of familiar and unfamiliar conspecific males.

Chapter III. This study explored the existence of sexual dichromatism in different colourful traits (shell and limb stripes) of Spanish terrapins. This study also examined the relationship between characteristics of coloration of the shell and limb stripes with body size and health-related physiological parameters (i.e., body condition, haematocrit and immune response) that could be related with dominance or quality of potential mates.

Chapter IV. This study explored how sex, body size and sexual coloration of Spanish terrapins affected inter-individual variability in boldness, as reflected by the antipredator hiding behaviour of withdrawing head and limbs into their own shell.

Chapter V. This study explored whether reproductive state of female terrapins *M. leprosa* imposes a cost in their boldness (i.e. antipredatory hiding responses into their shells) and/or exploratory behaviour in a novel-environment.

Chapter VI. This study explored in the field the relation of basking patterns of male terrapins, *M. leprosa*, with health state, body size and limb colour showiness.

Results

Role of chemical cues in mate choice and male intra-sexual recognition

The results obtained in **Chapter I** suggested that chemical signals may be involved in mate choice of the Spanish terrapins. However, female and male turtles had different responses when exposed to chemical cues of different conspecifics of the opposite sex. Females showed a preference for water pools with chemical cues of heavier males but the body condition or immune response of the male donor of the chemical cues were not important for their choice Otherwise, male *M. leprosa* preferred to occupy pools containing water with chemicals of healthy females (i.e. with greater immunocompetence), but pool selection did not depend of the body size and condition of the female donor of the chemical cues.

Concerning male interactions, **Chapter I** and **Chapter II** highlighted the importance of body size in chemosensory recognition between males. Male *M. leprosa* avoided water with chemicals of conspecific larger males, but oriented toward water with chemicals of smaller males. In addition, large but not small males spent less time in the pools with scent of unfamiliar males than in pools with scent of familiar males or with their own chemicals, which did not differ suggesting that only large unfamiliar males might represent a potential danger. In addition, **Chapter II** also provided evidence that male boldness affected their responses to chemical cues of familiar and unfamiliar male turtles. Shy individual male turtles spent more time in the pool with their own chemicals, avoiding pools with chemicals from either familiar or unfamiliar

males. In contrast, bold males preferred the chemicals of familiar males to the chemicals of unfamiliar males.

Function of sexual dichromatic coloration

Chapter III results showed the existence of chromatic differences between sexes of *M. leprosa* terrapins in the limb stripes but not in the shell. Females had limb stripes that were significantly brighter and with more ultraviolet (UV) saturated coloration than males. Moreover, characteristics of coloration of the limb stripes were related with body size and health state in females but not in males. Larger females had more UV saturated limb stripes coloration, and females with better immune responses had brighter limbs stripes. In contrast, shell coloration did not differ between sexes in *M. leprosa* terrapins, although some individual variations in shell coloration might mainly reflect ontogenetic changes in size-dependent predation risk. Larger males had darker and more UV saturated shell coloration and larger females also had more UV saturated shell coloration.

Sources of inter-individual variation in boldness under predation risk

Results obtained in the **Chapter IV** demonstrated that turtles were able to modify their antipredatory hiding behaviour in relation to the predation risk level. Thus, appearance and waiting times differed across three different levels of risk. Turtles had shorter appearance times when they were placed in a prone position ("low risk" treatment) than when they were overturned in the "medium risk" and "high risk" treatments. Similarly, when turtles were overturned, they had longer hiding times until their appeared from the

shell when risk increased as suggested by a longer time being handled by the predator ("high risk treatment") compared with the "medium risk" treatment. Concerning waiting times, turtles had longer waiting times in the "low risk" than in the "high risk" treatment. Differences between waiting times in the "medium risk" and "high risk" treatments only approached significance, and waiting times in the "low risk" and "medium risk" treatments did not differ.

In addition, boldness under predation risk was affected by several traits, such as gender, body size and coloration. Heavier turtles spent longer times hiding inside their shells after the attack during the "low risk" treatment (prone positioned) and started an active escape earlier than smaller ones in the "medium" risk treatment (overturned). The gender of the turtle also affected their appearance times during the "medium risk" treatment. Males spent less time than females inside their shells before they reappeared. In addition, brightness of limb stripes was related with appearance times in males, but not in females, and only when risk was low. Males with brighter coloration of limb stripes spent longer times hidden inside their shells in the "low" risk treatment.

Effect of reproductive state in exploratory activity and boldness

The results of **Chapter V** evidenced that some components of boldness (i.e. appearance times) but not exploratory activity of female turtles were affected by their reproductive state. In addition, boldness in appearance and waiting times were not related to boldness in exploratory behaviour of female terrapins. Thus, gravid turtles behave shier and had longer appearance times from inside their shells than non-gravid females in both the "low" and "high risk" treatments. Otherwise, reproductive state did not affect waiting times although gravid females with more eggs tended to have longer waiting times in

the “low” risk and shorter waiting times in the “high” risk level. Paradoxically, females carrying more eggs tended to have shorter waiting times under high risk, probably because in this context the turtle had assumed that had already been detected by the predator rendering unimportant other traits.

Basking behaviour, health state and colour conspicuousness

The results of the **Chapter VI** showed that basking activity was related with some variables that can reliable indicate the physiological-health state of the turtles. Similarly, colour showiness of the limb stripes in male Spanish terrapins affected their basking activity. Otherwise, body size did not influence basking behaviour of the turtles. Thus, male turtles with higher basking activity had lower total white blood cell (WBC) counts and lower values of haematocrit. In the same way, we found that male turtles that spent more time basking also had less bright coloration in limb stripes.

Conclusions

1. Spanish terrapins are able to communicate via chemical signals in water released by conspecifics. The results found here would suggest that male turtles may base their mating preferences on chemical cues that honestly report the female’s health state and, thus, invest in the quality of their future offspring. Otherwise, chemicals of males may convey information about their size to females and other males. Female choice of pools occupied by larger males could protect them from the harassment and forced inseminations of other males. On the other hand, in males, chemical detection and avoidance of chemicals of larger and stronger males may prevent agonistic

encounters with these males, in which smaller males could be at a disadvantage, allowing them to avoid injuries and save energy. In addition, male Spanish terrapins are able to distinguish between familiar and unfamiliar chemical cues found in the water. Similarly, male-male interactions in the Spanish terrapin could depend on the individual personality along the shy-bold axis and consequently affect their responses to conspecific chemicals. Taken together, these results suggest that male turtles might establish hierarchies during familiarization process, with bold males being dominant over shy ones. On the other hand, chemical cues from unfamiliar males might represent a hazard for both shy and bold males. Thus, boldness and body size could influence intrasexual competition between males.

2. Spanish terrapins show sexual dichromatism in limb but not in shell coloration. Moreover, limb coloration is more conspicuous in females than in males. Sexual selection forces could act strongly on female limb coloration promoting honest signals that are reliable for their receivers. Alternatively, duller coloration of limb strips in males might be linked to melanic changes related with age or size and to a high predation risk. In contrast, shell coloration does not differ between sexes, although it is related with body size in males and females. Smaller turtles have smaller amount of UV reflectance in their shells, which could render small turtles more difficult to detect by bird predators suggesting that shell coloration might have a cryptic function.

3. Individuals of *M. leprosa* are able to assess the predation risk level in their environment and optimise their antipredator hiding response. The results suggest that males and females differ in terms of the traits that influence their boldness in risky contexts in different ways and that different aspects of the antipredator behaviour might be influenced by different factors. Similarly, conspicuousness to predator eyes (i.e. larger size and brighter colours) might lead to differences in appearance times in

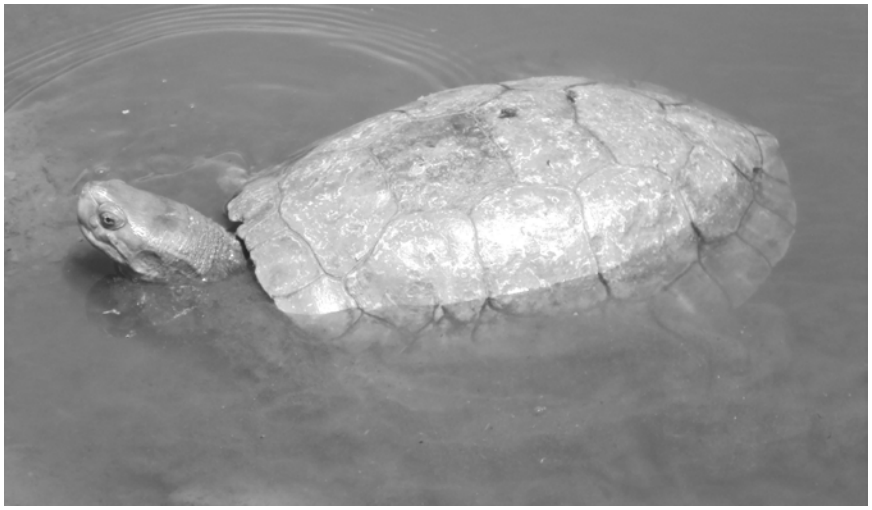
terrapins. Nevertheless, while the risk level increases, the turtles spend longer times inside their shells and need less time to initiate an active escape, and the relative contribution of size and coloration is less important (i.e. no relation in the “high” risk treatment). Thus, when risk increases, a turtle may assume that it has already been detected by the predator and then traits like body size or the degree of conspicuousness should not be important for their boldness in hiding behaviour, making the inter-individual differences in those traits irrelevant.

4. Hiding and exploratory behaviour are not related providing evidence that both traits are not a behavioural syndrome in female Spanish terrapins. The results obtained highlight that under risky situations, some components of boldness are affected by reproductive state of females. Gravid females appear relatively later from into their shells after a predatory attack than non-gravid ones. The lack of differences in relative and absolute weight between gravid and non-gravid females suggest that differences in appearance times might be due to metabolic-physiological costs and a worse condition of gravid females associated with egg production and embryo maintenance. However, gravid females might act more cautiously to protect their immediate higher fitness with respect to non-gravid ones.

5. Basking activity was related with health state and limb coloration. Male turtles that spent less time basking had higher total WBC counts and higher haematocrit values. Thus, males with higher basking activity could have less oxygen requirements due to their lower mobility and consequently reduced metabolical expenditure. Similarly, males that spent more time basking could be more susceptible to infection due to the fact that they dedicate less time to foraging which may compromise their immune system. Otherwise, basking behaviour could carry associate costs in terms of

predation risk with more conspicuous individuals reducing their basking activity to avoid be easily detected by potential predators.

Resumen



Introducción

Para comunicarse los animales puede utilizar una amplia gama de modalidades sensoriales tales como los sistemas táctil, químico, visual y auditivo.

En los reptiles, por un lado, la comunicación química intraespecífica tiene un papel crucial en la búsqueda pareja y selección de pareja, así como en la discriminación de la calidad de las parejas potenciales. Las señales químicas también son importantes en las relaciones entre machos, las cuales que pueden contribuir a reducir los costes derivados de las interacciones agresivas. Muchos estudios han demostrado que las tortugas tienen muy bien desarrollado tanto el sistema nasal como el vomeronasal, que se utilizan principalmente para discriminar diferentes olores, lo que sugiere una buena capacidad por parte de las tortugas para detectar señales químicas en el medio ambiente. Además, experimentos recientes en el laboratorio han demostrado la importancia de la comunicación química entre y dentro del mismo sexo en quelonios.

Por otro lado, los reptiles muestran coloraciones conspicuas durante la época reproductiva que también son importantes para la selección pareja y en las interacciones agonísticas entre machos. Varios estudios han demostrado la existencia de coloración dicromática sexual en algunas especies de tortugas. Sin embargo, sólo unos pocos estudios han demostrado que la coloración de las manchas y rayas situadas en la cabeza de las tortugas pueden estar relacionadas con la calidad individual, lo que sugiere que la coloración podría haber evolucionado bajo las fuerzas de selección sexual en tortugas.

Las señales sexuales van dirigidas a encontrar pareja con el beneficio de la reproducción por parte del emisor, lo que mejoraría eficacia biológica. Sin embargo, durante la emisión de las señales sexuales, los individuos también son más vulnerables a

ser localizados por los enemigos debido a su mayor vistosidad destinada a los receptores de tales señales. En este marco, las señales dirigidas a sus congéneres para la comunicación inter e intra-sexual pueden ser detectadas y explotadas por los depredadores. Además, las hembras reproductoras pueden estar más expuestas a los depredadores durante la etapa reproductiva debido a los cambios de comportamiento y fisiológicos relacionados con la reproducción. Similarmente, las hembras grávidas pueden mostrar una disminución de las capacidades locomotoras que podrían comprometer sus estrategias de escape cuando se enfrentan a un depredador. Sin embargo, a pesar de que los individuos con ornamentación extravagante, así como aquellos en una condición grávida reproductiva, deberían tener *a priori* un mayor riesgo de depredación, éstos podrían compensar su mayor vulnerabilidad con un comportamiento más prudente, y por lo tanto, reducir la presión predatoria a la que están sometidos.

En las regiones templadas, el principal peligro para las tortugas acuáticas proviene de depredadores terrestres, básicamente, mamíferos y aves. En este contexto, las tortugas tienen estructuras morfológicas, como el caparazón que ofrece una protección parcial (es decir, refugio) cuando se encuentran en hábitats terrestres, hasta que pueden escapar a un refugio más seguro sumergiéndose en el agua. Por lo tanto, las tortugas pueden retraer y esconder extremidades, cabeza y cola dentro del caparazón cuando un depredador se acerca demasiado y/o tratar de atacarlos. Sin embargo, aumentar el tiempo dedicado a esconderse dentro de un refugio o del caparazón, puede implicar una pérdida de oportunidades de apareamiento o de alimentación y también puede conllevar costes en términos de termorregulación. Por lo tanto, la decisión de cuándo salir del refugio e iniciar un escape activo puede dar lugar a un compromiso que los animales deberían resolver óptimamente mediante una modificación del tiempo

transcurrido dentro del refugio. El tiempo pasado en un refugio tiende a incrementar con los costes de emerger de tal refugio, atribuibles al riesgo de depredación, sugiriendo que la capacidad de evaluar el nivel de riesgo es fundamental para decidir cuándo dejar el refugio. El equilibrio entre los costes de permanecer escondido en un refugio contra los costes de salir de éste también debería depender de algunos rasgos como tamaño corporal, conspicuidad de la coloración sexual, estado reproductivo o estado de salud. Estos factores pueden explicar las variaciones interindividuales en la propensión a arriesgarse bajo riesgo de depredación, que a menudo se han utilizado para definir diferentes tipos de personalidad. De hecho, la selección natural, a través del régimen de la depredación, puede ser una de las principales fuerzas que modulan la evolución de personalidades.

Objetivos

Capítulo I. El presente estudio examinó las respuestas de comportamiento de los galápagos leprosos (*Mauremys leprosa*) a las señales químicas provenientes de individuos de la misma especie de diferente tamaño corporal y estado de salud. El primer experimento tuvo como objetivo examinar el papel potencial de las señales químicas en la elección de pareja. En un segundo experimento, se analizaron las respuestas de comportamiento de los machos a las señales químicas de otros machos.

Capítulo II. Este estudio examinó si los machos de *M. leprosa* pueden reconocer y distinguir a otros machos conocidos previamente y desconocidos a través de señales químicas liberadas en el agua. También se estudio la influencia del tamaño corporal y la personalidad de los machos en su respuesta a las señales químicas de otros machos conocidos y desconocidos de la misma especie.

Capítulo III. Este estudio exploró la existencia de dicromatismo sexual en la coloración de diferentes zonas del cuerpo (caparazón y rayas de las extremidades) de galápago leproso. Este estudio también examinó la relación entre las características de coloración de las rayas de las extremidades y del caparazón con el tamaño corporal y parámetros fisiológicos y estado de salud (condición corporal, hematocrito y respuesta inmune) que podrían estar relacionados con la dominancia entre congéneres o la calidad de la pareja.

Capítulo IV. Este estudio exploró cómo sexo, tamaño corporal y coloración sexual de los galápagos afectó a la variabilidad interindividual en su propensión a arriesgarse bajo riesgo de depredación, reflejado como el tiempo que los galápagos pasan dentro de su caparazón hasta iniciar una estrategia de escape activo después del ataque de un depredador.

Capítulo V. Este estudio exploró si el estado reproductivo de las hembras de *M. leprosa* impuso un coste en el comportamiento antidepredatorio (es decir, el tiempo transcurrido dentro de sus caparazones después del ataque de un depredador) y/o en el comportamiento exploratorio de las mismas.

Capítulo VI. Este estudio abordó, en el campo, la relación entre los patrones de asoleamiento de tortugas macho de *M. leprosa*, con su estado de salud, tamaño corporal y vistosidad de su coloración.

Resultados

Papel de señales químicas en la elección de pareja y el reconocimiento entre machos

Los resultados obtenidos en el **Capítulo I** sugieren que las señales químicas pueden estar involucradas en la elección de pareja de los galápagos leprosos. Sin embargo, las tortugas de ambos sexos tuvieron respuestas diferentes cuando se expusieron a las señales químicas de los congéneres del sexo opuesto. Las hembras mostraron una preferencia por las piscinas que contenían agua con señales químicas de machos más grandes, pero la condición corporal o la respuesta inmune del macho donante de las señales químicas no fueron importantes para su elección. Por el contrario, los machos de *M. leprosa* prefirieron ocupar piscinas que contenían agua con señales químicas de hembras más sanas (es decir, con mayor inmunocompetencia), pero la selección de las piscinas no dependió del tamaño ni la condición de la hembra de la que provenían tales señales químicas.

En cuanto a las interacciones entre machos, los **Capítulos I** y **II** destacaron la importancia del tamaño corporal en el reconocimiento químico entre machos. Los machos de *M. leprosa* evitaron el agua con olor de machos más grandes, pero mostraron una preferencia hacia el agua con señales químicas de machos más pequeños. Además, los machos grandes, pero no los pequeños, pasaron menos tiempo en las piscinas con el olor de los machos desconocidos comparado con el tiempo que pasaron en piscinas con olor de machos conocidos previamente o con sus propio olor, lo que sugiere que sólo los machos grandes desconocidos podrían representar un peligro potencial. Además, el **Capítulo II** también puso en evidencia que la personalidad de los machos afectó a su respuesta quimiosensorial de otros machos conocidos y desconocidos. Los machos clasificados con una personalidad tímida pasaron más tiempo en las piscina que contenían su propio olor, y evitaron ocupar piscinas con señales químicas de cualquier macho, ya sea conocido o desconocido. Por el contrario, los galápagos clasificados

como atrevidos prefirieron el olor de machos conocidos respecto a las señales químicas de otros machos no conocidos previamente.

Dicromatismo sexual y función de la coloración

Los resultados del **Capítulo III** mostraron la existencia de diferencias cromáticas entre sexos en las extremidades, pero no en el caparazón, de los galápagos. Las hembras presentaron rayas de las extremidades que fueron significativamente más brillantes y con una coloración ultravioleta (UV) más saturada que los machos. Por otra parte, las características de coloración de las rayas de las extremidades estuvieron relacionadas con el tamaño corporal y el estado de salud en las hembras pero no en los machos. Aquellas hembras más grandes tuvieron una coloración UV más saturada en sus extremidades. De la misma manera, las hembras con mejor respuesta inmune tuvieron rayas de las extremidades más brillantes. Por el contrario, la coloración del caparazón no difirió entre sexos en los galápagos, aunque algunas variaciones individuales en la coloración del caparazón podrían reflejar principalmente los cambios ontogénicos en el riesgo de depredación, dependientes del tamaño individual. Los machos de mayor tamaño presentaron caparazones mas oscuros y con coloración UV más saturada y las hembras más grandes también tuvieron caparazones con un mayor nivel de UV.

Fuentes de variación interindividual en la propensión a arriesgarse bajo situaciones de peligro

Los resultados obtenidos en el **Capítulo IV** demuestran que las tortugas fueron capaces de regular la estrategia del uso del caparazón como refugio (es decir, la propensión a arriesgarse en situaciones de peligro) en relación con el nivel de riesgo de depredación. Por lo tanto, los tiempos de aparición y de espera hasta iniciar un escape activo por parte de las tortugas difirieron en los tres niveles de riesgo. Los galápagos tuvieron tiempos de aparición más cortos cuando fueron colocados boca abajo, sobre su propio plastrón (tratamiento de "riesgo bajo"), que cuando se les colocó volcados sobre su propio caparazón, en los tratamientos de "riesgo medio" y de "riesgo alto". Del mismo modo, cuando las tortugas fueron volcadas, pasaron más tiempo escondidas dentro del caparazón hasta que aparecieron a medida que el riesgo incrementó, es decir, cuando fueron sujetadas durante más tiempo por el depredador (tratamiento de “riesgo alto”) en comparación con el tratamiento de “riesgo medio”. En cuanto a los tiempos de espera, las tortugas tuvieron tiempos de espera hasta iniciar un escape activo más largos en el tratamiento de “riesgo bajo” que en el tratamiento de “riesgo alto”. Las diferencias entre los tiempos de espera en los tratamiento de "riesgo medio" y de "riesgo alto" tendieron a la significación, y los tiempos de espera en los tratamiento de “riesgo bajo” y de "riesgo medio” no difirieron.

Además, la propensión a arriesgarse bajo riesgo de depredación se vio afectada por varias características, tales como el sexo, el tamaño corporal y la coloración de los galápagos. Las tortugas más pesadas pasaron tiempos más largos dentro de sus caparazones después de un ataque simulado durante el tratamiento de "riesgo bajo" (posición sobre su plastrón) y comenzaron un escape activo antes que los más pequeños

en el tratamiento de “riesgo medio” (posición volcada). El sexo de los galápagos también afectó a sus tiempos de aparición durante el tratamiento de "riesgo medio". Los machos pasaron menos tiempo que las hembras dentro de sus caparazones antes de reaparecer. Además, el grado de conspicuidad en las extremidades estuvo relacionado con tiempos de aparición en machos, pero no en hembras, y sólo cuando el riesgo fue bajo. De esta manera, los machos con una coloración más brillante en las manchas de sus extremidades pasaron más tiempo escondidos dentro de sus conchas hasta que reaparecieron en el tratamiento de “riesgo bajo”.

Efecto del estado reproductivo en la propensión a arriesgarse y la actividad exploratoria

Los resultados del **Capítulo V** evidenciaron que algunos componentes del comportamiento antidepredatorio (es decir, tiempos de aparición), pero no la actividad exploratoria, de las tortugas hembra se vieron afectadas por su estado reproductivo. Además, tanto los tiempos de aparición como los tiempos de espera después de un ataque no estuvieron relacionados con el comportamiento exploratorio de los galápagos. Por lo tanto, las tortugas grávidas se comportaron más tímidamente, y tuvieron tiempos de aparición más largos hasta que emergieron de sus caparazones que las hembras no grávidas, tanto en los tratamientos de riesgo “bajo” y de riesgo “alto”. De lo contrario, el estado reproductivo no afectó a los tiempos de espera, aunque las hembras grávidas con más huevos tendieron a tener mayores tiempos de espera en el tratamiento de riesgo “bajo” y tiempos de espera más cortos en el tratamiento de riesgo “alto”. Paradójicamente, las hembras con una mayor carga de huevos tendieron a tener tiempos de espera más cortos cuando el riesgo fue alto, probablemente porque en este

contexto la tortuga ya ha asumido que ha sido detectada por el depredador, dejando sin importancia otros rasgos individuales.

Comportamiento de asoleamiento, estado de salud y conspicuidad de la coloración

Los resultados del **Capítulo VI** mostraron que la actividad de asoleamiento se relacionó con algunas variables que pueden indicar el estado fisiológico y de salud de las tortugas. Del mismo modo, la vistosidad de la coloración de las rayas de las extremidades en machos de galápago leproso afecto a su patrón de actividad de asoleamiento. De lo contrario, el tamaño corporal no influyó en el comportamiento de asoleamiento. Por lo tanto, las tortugas machos con mayor actividad de asoleamiento tuvieron recuentos de glóbulos blancos (WBC) más bajos y valores de hematocrito menores. También obtuvimos que los machos que pasaron más tiempo asoleándose presentaron una coloración más oscura en sus extremidades.

Conclusiones

1. Los galápagos leprosos son capaces de comunicarse a través de señales químicas liberadas al agua por sus congéneres. Los resultados obtenidos en la presente tesis sugieren que los galápagos macho pueden basar sus preferencias de apareamiento en las señales químicas que informan honestamente del estado de salud de las hembras y, por lo tanto, invertir en la calidad de su descendencia futura. De lo contrario, las señales químicas de los machos pueden transmitir información sobre su tamaño para las hembras y otros machos. La elección de piscinas ocupadas por machos más grandes por parte de las hembras podría protegerlas de la persecución y las inseminaciones forzadas

de otros machos. Por otra parte, en los machos, la detección y evitación de las sustancias químicas de machos más grandes y fuertes podría impedir encuentros agonísticos con tales machos, situación en la cual los machos más pequeños podrían estar en desventaja, lo que les permitiría evitar lesiones y ahorrar energía. Además, los machos de galápago leproso son capaces de distinguir entre señales químicas de otros machos conocidos y no conocidos previamente. Del mismo modo, las interacciones entre machos de galápago leproso podrían depender de la "personalidad" individual a lo largo del eje tímido-atrevido y en consecuencia a su discriminación química. Tomados en conjunto, estos resultados sugieren que los machos de esta especie pueden establecer jerarquías durante el proceso de familiarización, con machos más atrevidos dominando sobre los tímidos en sus interacciones. Por otro lado, las señales químicas de los machos desconocidos pueden representar un peligro tanto para los machos tímidos como atrevidos. Por lo tanto, la "personalidad" y el tamaño corporal podrían influir en la competencia entre machos.

2. Los galápagos leprosos presentan dicromatismo sexual en la coloración de las extremidades, pero no del caparazón. Además, la coloración de las extremidades es más conspicua en hembras que en machos. De esta manera, las presiones ejercidas por la selección sexual podrían actuar fuertemente sobre la coloración de las extremidades en las hembras promoviendo señales honestas que sean fiables para sus receptores. Por otra parte, la coloración más apagada en las rayas de las extremidades de los machos podría estar vinculada a cambios melánicos relacionados con la edad o el tamaño y con un alto riesgo de depredación. En contraste, la coloración del caparazón no difiere entre sexos, aunque está relacionada con el tamaño del cuerpo en machos y hembras. Las tortugas de menor tamaño tienen menor cantidad de saturación UV en sus caparazones, lo que podría hacer que las tortugas más pequeñas fueran más difíciles de detectar por las aves

depredadoras lo que sugieren que la coloración del caparazón podría tener una función críptica.

3. Los individuos de *M. leprosa* son capaces de evaluar el nivel de riesgo de depredación en su entorno y optimizar su estrategia antidepredatoria, como el tiempo pasado dentro del caparazón. Los resultados sugieren que machos y hembras difieren en cuanto a los rasgos que influyen en su propensión a arriesgarse en situaciones de peligro y que los diferentes aspectos del comportamiento antidepredador pueden estar influenciados por diferentes factores. Del mismo modo, una mayor vistosidad hacia los depredadores (es decir, un tamaño mayor y colores más brillantes) podría dar lugar a diferencias en los tiempos de aparición. Sin embargo, mientras que aumenta el nivel de riesgo, las tortugas pasan tiempos más largos dentro de sus conchas y necesitan menos tiempo para iniciar un escape activo y la contribución relativa del tamaño y la coloración es menos importante (es decir, sin relación alguna en el tratamiento de riesgo "alto"). Por lo tanto, cuando aumenta el riesgo, un galápago puede asumir que ya ha sido detectado por el depredador y que sus rasgos individuales, tales como el tamaño corporal o el grado de conspicuidad, no deberían ser importante para su propensión a arriesgarse.

4. La propensión a arriesgarse bajo riesgo de depredación y la propensión a arriesgarse en un nuevo ambiente (comportamiento exploratorio) no están relacionados proporcionando pruebas de que ambos rasgos no son un síndrome conductual en hembras de galápago leproso. Los resultados obtenidos ponen de manifiesto que bajo situaciones de riesgo, algunos componentes del comportamiento antidepredatorio se ven afectados por el estado reproductivo de las hembras. Las hembras grávidas aparecen relativamente más tarde de sus caparazones (tiempos de aparición mayores) después del ataque de un depredador que las hembras no grávidas. La falta de diferencias en el peso

los tiempos de aparición podrían deberse a los costes metabólicos, fisiológicos y una peor condición de las hembras grávidas asociados a la producción de huevos y el mantenimiento de los embriones. Sin embargo, las hembras grávidas pueden actuar con más cautela para proteger su mayor eficacia biológica (“fitness”) inmediata con respecto a las no grávidas.

5. La actividad de asoleamiento estuvo relacionada con el estado de salud y la coloración de las extremidades. Los galápagos macho que pasaron menos tiempo asoleándose tuvieron un mayor recuento total de leucocitos y valores superiores de hematocrito. Por lo tanto, los machos con una mayor actividad de asoleamiento podrían gastar menos oxigeno, debido a su menor movilidad y en consecuencia tener un gasto metabólico reducido. Asimismo, los machos que pasaron más tiempo asoleándose podrían ser más susceptibles a las infecciones debido al hecho de que dedican menos tiempo a la búsqueda de alimento, hecho que podría comprometer su sistema inmunológico. De lo contrario, el comportamiento de asoleamiento podría llevar costes asociados en términos de riesgo de depredación, con individuos más conspicuos reduciendo su actividad de asoleamiento para evitar ser detectados fácilmente por los depredadores potenciales.